

Analytical Profiles of Drug Substances

Volume 10

Edited by

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PREFACE

Although the official compendia list tests and limits for drug substances related to identity, purity, and strength, they normally do not provide other physical or chemical data, nor do they list methods of synthesis or pathways of physical or biological degradation and metabolism. For drug substances important enough to be accorded monographs in the official compendia, such supplemental information should also be made readily available. To this end the Pharmaceutical Analysis and Control Section, Academy of Pharmaceutical Sciences, has undertaken a cooperative venture to compile and publish *Analytical Profiles of Drug Substances* in a series of volumes of which this is the tenth.

The concept of analytical profiles is taking hold not only for compendial drugs but, increasingly, in the industrial research laboratories. Analytical profiles are being prepared and periodically updated to provide physiochemical and analytical information of new drug substances during the consecutive stages of research and development. Hopefully, then, in the not-too-distant future, the publication of an analytical profile will require a minimum of effort whenever a new drug substance is selected for compendial status.

The cooperative spirit of our contributors has made this venture possible. It is gratifying to note that increasingly profiles are being written not only in industrial laboratories but also in academic institutions worldwide.

All those who have found the profiles useful are requested to contribute a monograph of their own. The editors stand ready to receive such contributions.

The goal to cover all drug substances with comprehensive monographs is still a distant one. It is up to our perseverance to make it a reality.

Klaus Florey

AMINOSALICYLIC ACID

*Mahmoud M. A. Hassan, Ahmad I. Jado,
and Muhammad Uppal Zubair*

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1. DESCRIPTION

1.1 Nomenclature

1.1 1 Chemical Names

- a. 4-Amino-2-hydroxybenzoic acid.
- b. 4-Aminosalicylic acid.
- c. Benzoic acid, 4-Amino-2-hydroxy.

The CAS Registry No. is [65-49-6].

1.1 2 Generic Name

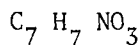
p-Aminosalicylic acid.

1.1 3 Trade Names

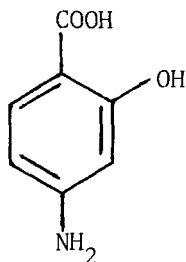
Apas, Apacil, Deapasil, Hellipidyl, PAS, . PAS-C, Pamcyl, Pamisyl, Parasil, Pasorbic, Pasolac, Parasalicil, Parasalindon, Pasnodia, Propasa, Rezipas, Sanipirrol-4, Para-Pas, Pasem.

1.2 Formulae

1.2 1 Empirical



1.2 2 Structural



1.2 3 Wiswesser Line Notation

ZR CQ DVQ

1.3 Molecular Weight

153.13

1.4 Elemental Composition

C, 54.90%; H, 4.61%; N, 9.5%; O, 31.34%.

1.5 Appearance, Color, Taste, Odor

White, or yellowish white, bulky powder or crystals darkens on exposure to light and air, odorless or has slight acetous odor.

2. Physical Properties

2.1 Crystal Properties

2.1.1 X-Ray Diffraction

Crystal data

Monoclinic, $a = 7.209$ (2), $b = 3.786$ (1), $c = 25.109$ (9) Å, $B = 103.22$ (3)°, $U = 6.67.14$ Å³, $Z = 4$, $D_c = 1.53$, $F(000) = 320$. Cu-Kα radiation, $\lambda = 1.5418$ Å; μ (Cu-Kα) = 10.20cm^{-1} . Systematic absences = $h0l$, $l = 2n + 1$, $0k0$, $k = 2n + 1$, space group $P2_1/C$ from systematic absences (1).

Optical goniometry

It crystallises from ethanol in at least two habits. The interfacial angles of habit I were measured with a Huber two circle optical goniometer and compared with angles calculated from unit-cell dimensions for all faces having Miller indices between (and including) +2 and -2. A unique set of assignments for the faces was obtained and confirmed by precision photography. The $h k o$ net was in approximately reflecting position on the precession camera when the face-assigned indices (001) were approximately normal to X-ray beam. Fig. 1 shows a schematic drawing of habit I with assigned faces. The end faces of habit II did not have the indices (011) but precession photography and optical goniometry showed that (001) and (10?) were its two largest faces.

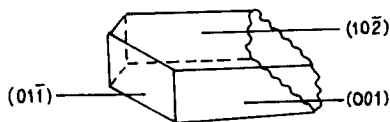
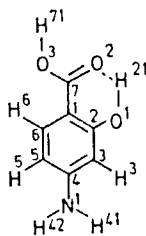


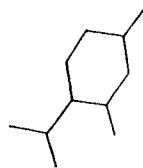
Fig. 1 : Schematic diagram of crystals of p-Aminosalicylic acid in habit I.

Crystal Structure

Two different crystal structures have been reported for p-aminosalicylic acid. Structure II has been reported before the advent of modern computers (2) while structure I has been developed very recently (1). Table 1 and 2 list the bond lengths and angles and Table 3 atom positions. Intramolecular contacts and angles involving the $O(1)-H(21)\dots O(2)$ hydrogen bond are also included. Data for p-aminosalicylic acid are consistent with the idea that resonance structure (Ib) and (Ic) contribute significantly to its structure.



I



II

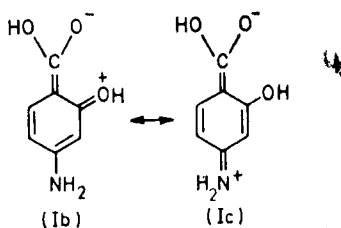


Table 1

Bond lengths (Å) in p-aminosalicylic acid (1), with standard deviations in parentheses. Intramolecular contacts involving the O(1)-H(21)...O(2) hydrogen bond are included.

O(1)-C(2)	1.361(2)	C(1)-C(2)	1.414(2)
O(2)-C(7)	1.243(2)	C(1)-C(6)	1.400(3)
O(3)-C(7)	1.311(2)	C(1)-C(7)	1.447(2)
O(2)...O(1)	2.620(2)	C(2)-C(3)	1.371(2)
N(1)-C(4)	1.364(2)	C(3)-C(4)	1.392(3)
O(1)-H(21)	0.98(3)	C(4)-C(5)	1.406(3)
O(3)-H(71)	0.95(3)	C(5)-C(6)	1.362(2)
O(2)...H(21)	1.73(3)	C(3)-H(3)	0.98(2)
N-H(41)	0.91(3)	C(5)-H(5)	0.98(2)
N-H(42)	0.83(3)	C(6)-H(6)	0.94(2)

Table 2

Bond angles(°) in p-aminosalicylic acid (1), with estimated standard deviations in parentheses. Angles involving the O(1)-H(21)...O(2) hydrogen bond are included.

O(2)-C(7)-O(3)	121.1(1)	O(1)-C(2)-C(3)	118.2(2)
O(2)-C(7)-C(1)	123(2)	C(2)-C(3)-C(4)	121.1(2)
O(3)-C(7)-C(1)	115.8(2)	C(3)-C(4)-C(5)	118.7(1)
C(7)-C(1)-C(2)	120.8(2)	C(3)-C(4)-N(1)	120.7(2)
C(7)-C(1)-C(6)	121.7(2)	C(5)-C(4)-N(1)	120.6(2)
C(2)-C(1)-C(6)	117.4(1)	C(6)-C(5)-C(4)	120.1(2)
C(1)-C(2)-O(1)	121.3(1)	C(1)-C(6)-C(5)	122.0(2)
C(1)-C(2)-C(3)	120.6(2)		
H(71)-O(3)-C(7)	113(2)	H(41)-N(1)-C(4)	120(2)
H(21)-O(1)-C(2)	107(2)	H(42)-N(1)-C(4)	115(2)
O(2)...H(21)-O(1)	147(3)	H(5)-C(5)-C(4)	119(1)
C(7)-O(2)...H(21)	100(1)	H(5)-C(5)-C(6)	121(1)
H(3)-C(3)-C(2)	118(1)	H(6)-C(6)-C(1)	119(1)
H(3)-C(3)-C(4)	121(1)	H(6)-C(6)-C(5)	119(1)
H(41)-N(1)-H(42)			

Table 3

Final atomic positions ($\times 10^4$; for H $\times 10^3$) for p-amino-salicylic acid (I), with standard deviations in parentheses.

	x	y	z
O(1)	6 882(2)	3 539(4)	1 641.0(5)
O(2)	5 572(2)	1 178(4)	651.0(5)
O(3)	7 438(2)	1 345(4)	58.2(5)
N(1)	13 290(3)	7 453(5)	2 111.6(8)
C(1)	8 718(2)	3 353(5)	946.6(6)
C(2)	8 539(2)	4 138(5)	1 483.1(6)
C(3)	10 041(3)	5 531(5)	1 860.5(7)
C(4)	11 784(2)	6 175(5)	1 728.6(7)
C(5)	11 966(3)	5 457(5)	1 193.8(7)
C(6)	10 474(2)	4 058(5)	819.9(7)
C(7)	7 136(2)	1 880(5)	547.1(6)
H(21)	601(4)	241(9)	133(1)
H(71)	637(4)	37(8)	-19(1)
H(41)	1 316(4)	789(8)	246(1)
H(42)	1 427(4)	782(8)	200(1)
H(3)	958(3)	602(6)	223(1)
H(5)	1 319(3)	595(6)	110(1)
H(6)	1 064(2)	354(5)	47(1)

2.1 2 Melting Range

The melting point of 4-aminosalicylic acid is uncertain (3) : 135°-140° with decomposition (4), 148° (dec.) (5), 149-151° (dec.) (6). 150-151° with effervescence (7,8), 139-141° (dec.) (9) and 220° (dec.) (10,11) have been reported. Seaman *et al* (3) have concluded that the most nearly correct melting point is about 240° and the melting point is not a good criterion of purity.

2.2 Solubility

1 g in about 600 ml of water and about 21 ml of alcohol; slightly soluble in ether; practically insoluble in benzene. Solubility is increased with alkaline salts of alkali metals (NaHCO_3) and in weak nitric acid, the amine salts of hydrochloric and sulphuric acids are insoluble. The aqueous solutions have a pH of about 3.2 and when heated the acid decomposes (12).

2.3 Identification

1. p-Aminosalicylic acid gives an intense orange-brown color when reacted with potassium ferricyanide in alkaline solution (13).
2. It gives a green color which changes first to orange and then to orange-red on reaction with hexamine and sulphuric acid at room temperature (14).

2.4 Spectral Properties

2.4 1 Infrared Spectrum

The infrared spectrum of 4-aminosalicylic acid is recorded as a nujol mull on Unicam SP 1025 Spectrophotometer and is shown in Fig. 2. The assignments for the characteristic bands in the infrared spectrum listed in Table 4.

Table 4

<u>Frequency cm⁻¹</u>	<u>Assignment</u>
3520	NH ₂
3400	NH ₂ , OH
1630	bonded C = O
890	isolated C-H out of plane deformation.
820	C-H out of plane deformation.
800	
770	

Other characteristic finger print bands are:

1305, 1230, 1200, 1170, 1110, 970, 725 and 690 cm⁻¹. Other values for PAS in potassium bromide disc (15) are, 3571, 3448, 3030, 1667, 1613, 1515, 1449, 1299, 1220, 1190, 1163, 813 and 775.

2.4 2 Ultraviolet Spectrum (UV)

UV spectrum of PAS in ethanol was scanned using Cary, 219 spectrophotometer ; from 400 to 200 nm(16), three maxima and two minima were observed. The maxima are located at 235, 274 and 303 nm.

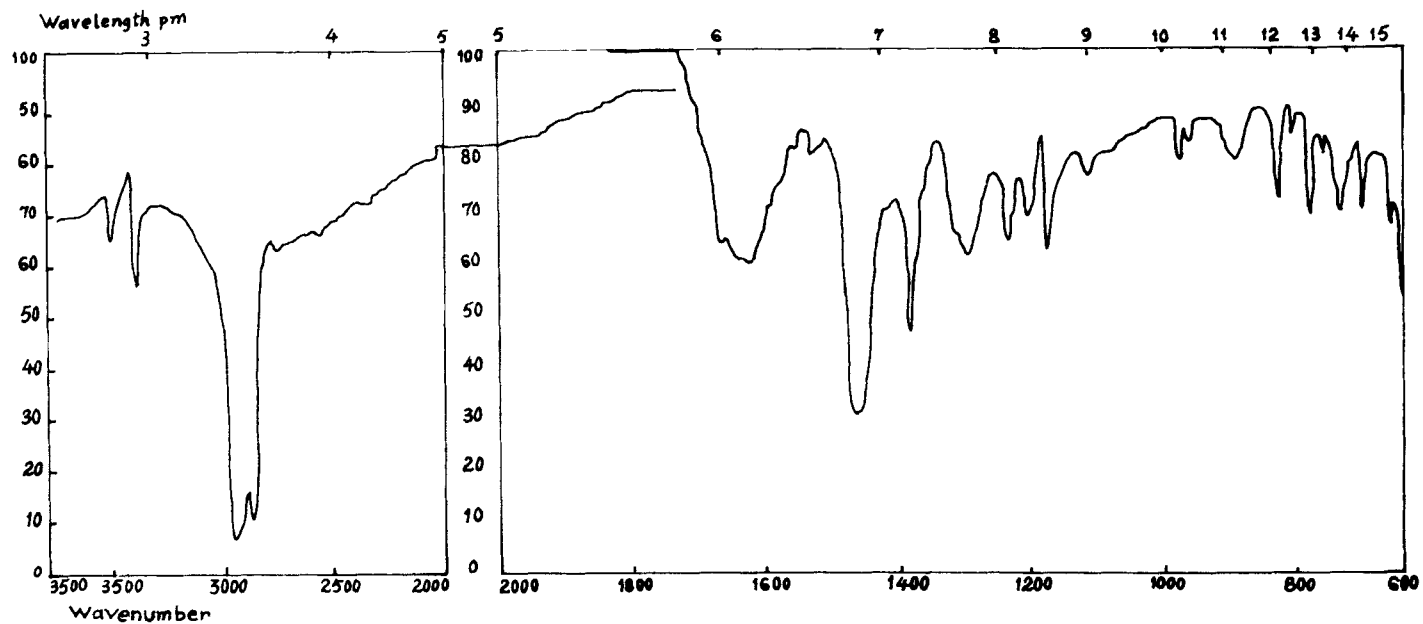


Fig. 2 : Infrared Spectrum of p-Aminosalicylic acid in Nujol.

The minima occur at 252 and 289 nm. The spectrum is shown in Fig. 3. The UV spectral data of PAS have also been reported earlier (17).

2.4 3 Nuclear Magnetic Resonance Spectrum (NMR)

PMR

The proton NMR spectra of PAS in DMSO-d₆ and in acetone-d₆ are shown in Fig. 4 and 5. These were recorded on Varian T-60A, 60 MHz NMR Spectrometer, using tetramethylsilane as internal reference (18). The PMR spectral data of PAS are given in Table 5.

Table 5 : PMR Chemical Shifts of PAS

	Chemical shifts (δ)					
	C(1) - CO (s)	OH C(2)- (s)	C(3) - H (s)	C(4)-NH ₂ (s)	C(5)-H (d)	C(6)-H
DMSO-d ₆	8.07	8.07	6.08	6.08	6.13	7.50
Acetone-d ₆	-	-	6.10	6.10	6.20	7.56

(s) = singlet, (d) = doublet.

Long range coupling between the C(3)-H and C(5)-H is observed in the 200 MHz spectrum in DMSO-d₆ (Fig. 6) (18).

¹³C NMR

Hassan and Uppal Zubair (19) have investigated the ¹³C NMR spectrum of PAS, and determined its carbon shifts. The spectrum (Fig. 7) shows seven singlets. The carbon chemical shifts of PAS in hexadeuterodimethylsulfoxide are as follows:

CO : 172.17, C(1) : 100.46, C(2) : 163.56, C(3) : 98.81, C(4) : 155.73, C(5) : 106.34, C(6) : 131.56.

The off-resonance decoupled spectrum Fig.8 and 9 shows four singlets representing CO, C(1), C(2)

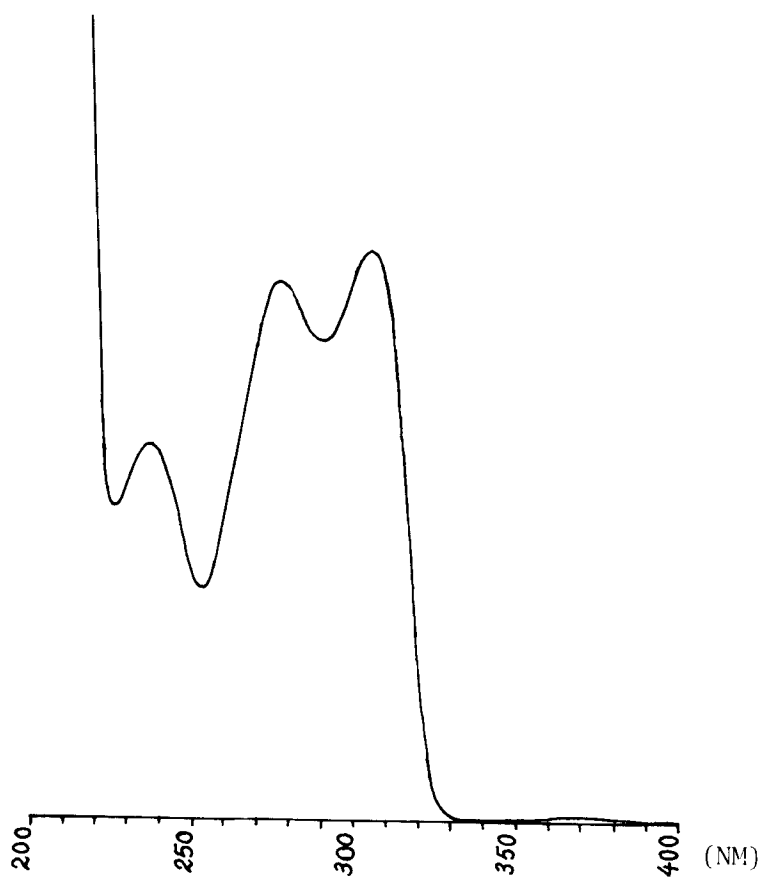


Fig. 3 : UV Spectrum of p-Aminosalicylic acid in Ethanol.

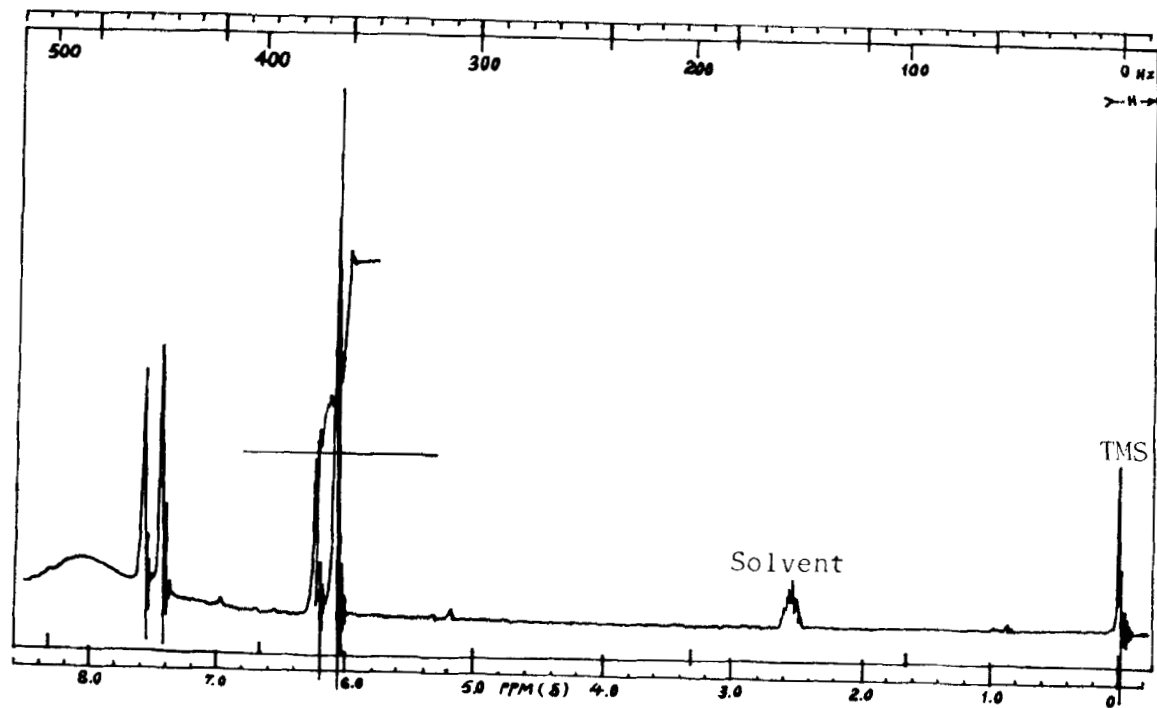


Fig. 4 : PMR Spectrum of p-Aminosalicylic acid in DMSO-d₆ and TMS.

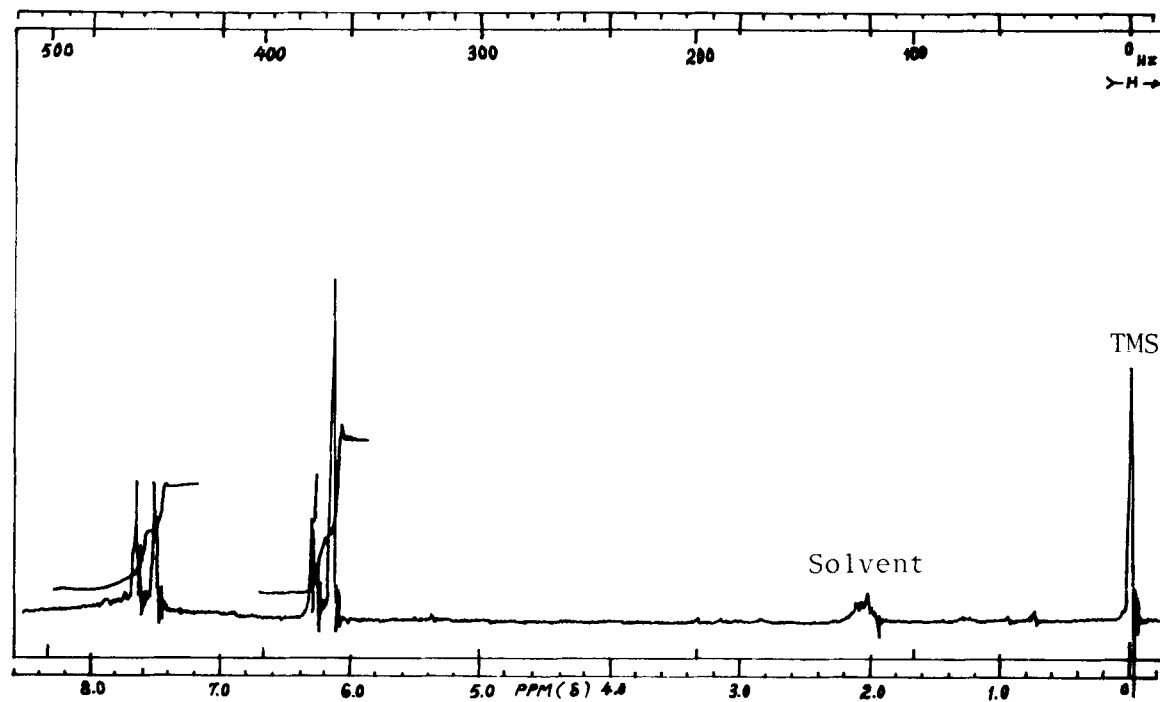


Fig. 5 : PMR Spectrum of p-Aminosalicylic acid in Acetone- d_6 and TMS.

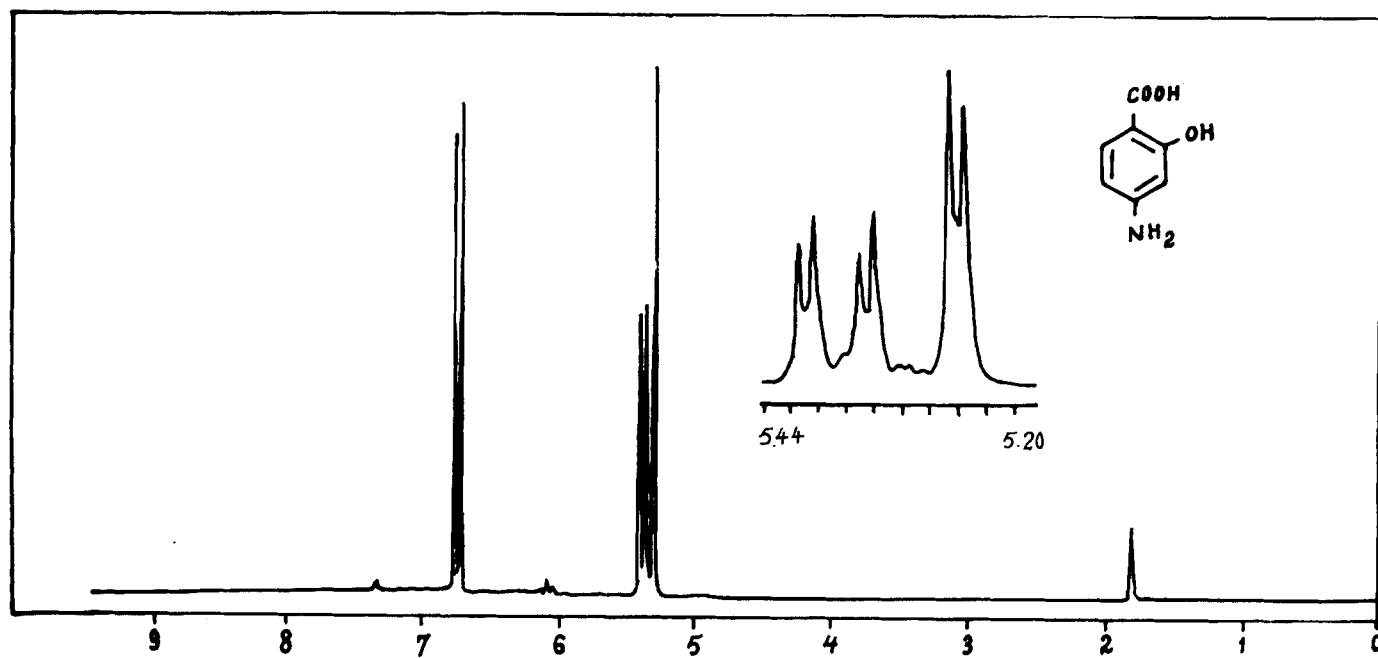


Fig. 6 : 200 MHz PMR Spectrum of p-Aminosalicylic acid in DMSO-d₆.

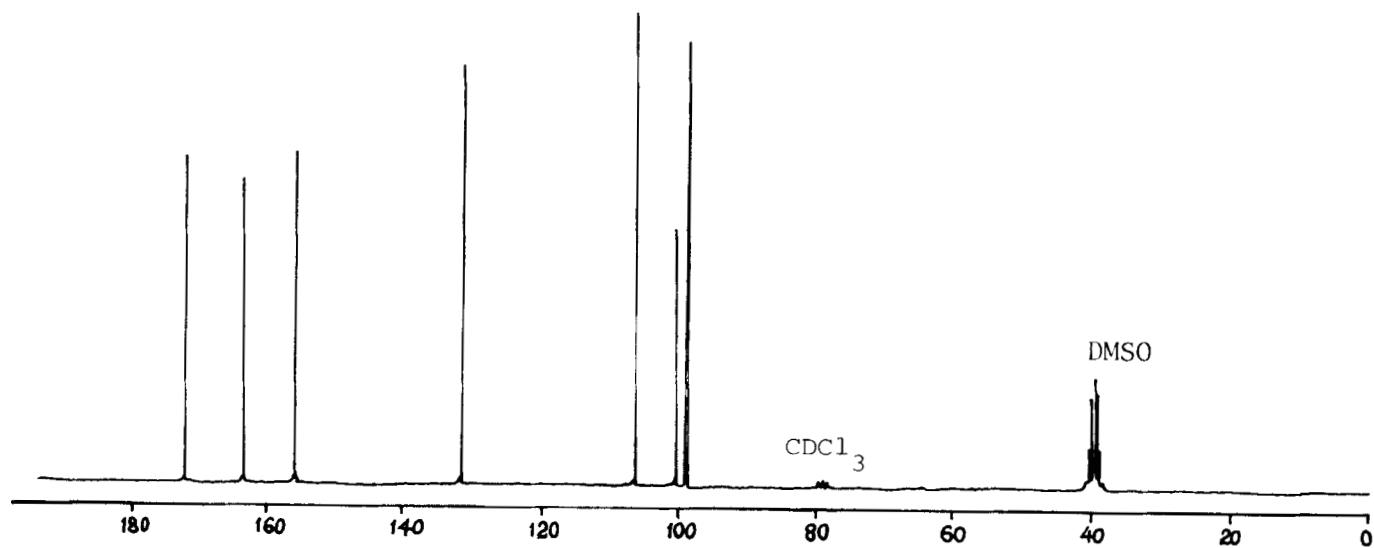


Fig. 7 : ^{13}C NMR Spectrum of p-Aminosalicylic acid in DMSO-d_6 .

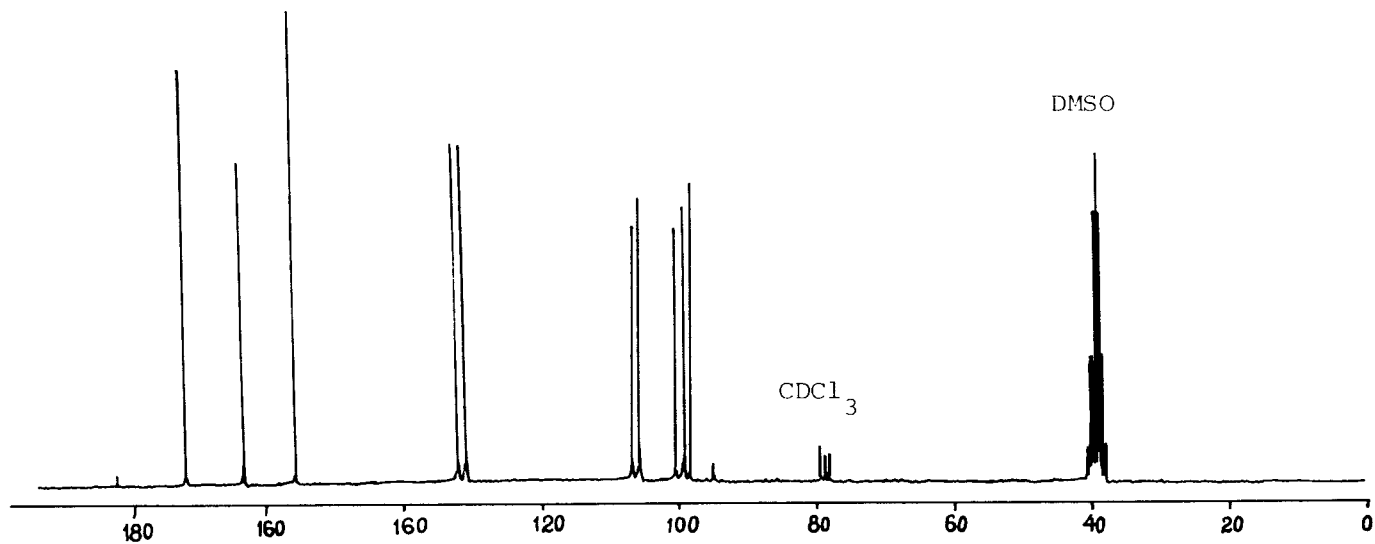


Fig. 8 : ^{13}C NMR off Resonance Decoupled Spectrum of p-Aminosalicylic acid in DMSO-d_6 .

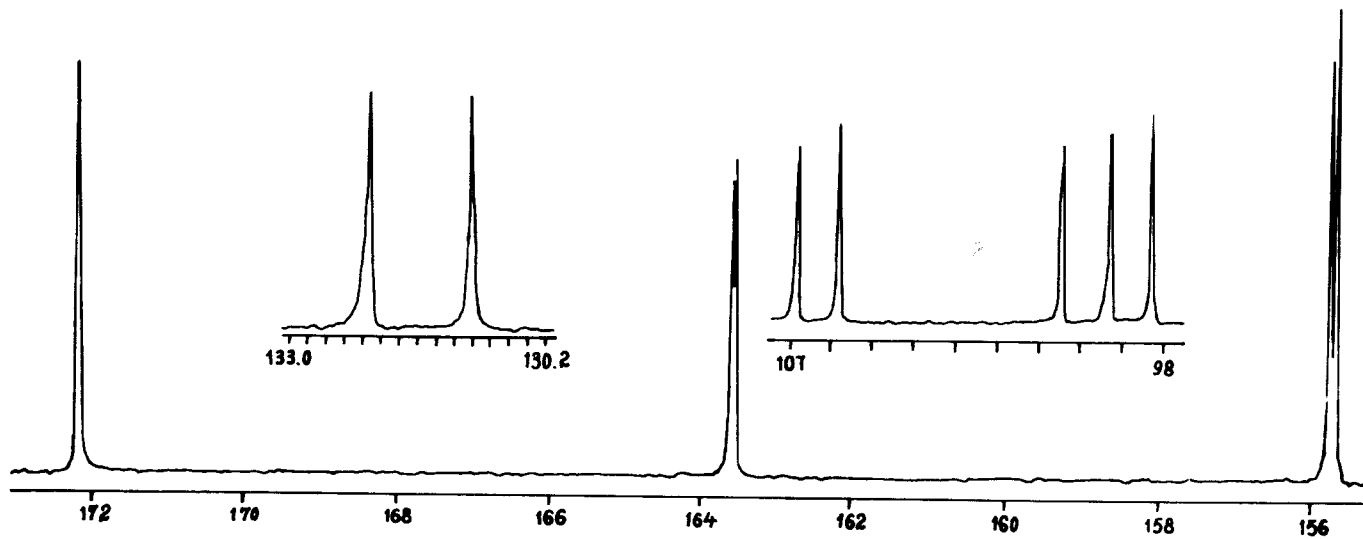


Fig. 9 : Part of ^{13}C NMR off Resonance Decoupled Spectrum of p-Aminosalicylic acid (expanded).

and C(4) and three doublets representing C(3), C(5) and C(6). The carbon chemical shifts are as follows:

CO : 172.08, C(1) : 100.49, C(2) : 163.59,
C3 : 99.29, and 98.33, C(4) : 155.77, C(5) :
106.84 and 105.85, C(6) : 132.12 and 131.03.

2.4 4 Mass Spectrum

The mass spectrum of PAS obtained by conventional electron impact ionisation shows a molecular ion M^+ at m/e 153. The base peak is at m/e 135. The M^+ ion peak has about 62.1% relative intensity (Fig.10). The m/e for the most prominent fragments are listed in Table 6. Tatematsu et al, have also reported the mass spectrometry of mixed drugs including 4-aminosalicylic acid (20).

Table 6.

m/e	Relative Intensity
52	14.3
79	14.3
107	24.6
135	100.0
136	15.1
153	62.1

3. Synthesis

Several synthetic routes to 4-aminosalicylic acid have been reported (21-30). Two of these are illustrated below.

Route I: Modified Kolbe-Schmidt Reaction of 4-aminosalicylic acid have been obtained by heating dry finely divided *m*-aminophenol and potassium carbonate under anhydrous conditions at 150-190° in CO₂ atmosphere (27). The yield is 90%.

Route II: This route describes the synthesis of C¹⁴-carboxyl-labelled 4-aminosalicylic acid by Sandmeyer Reaction

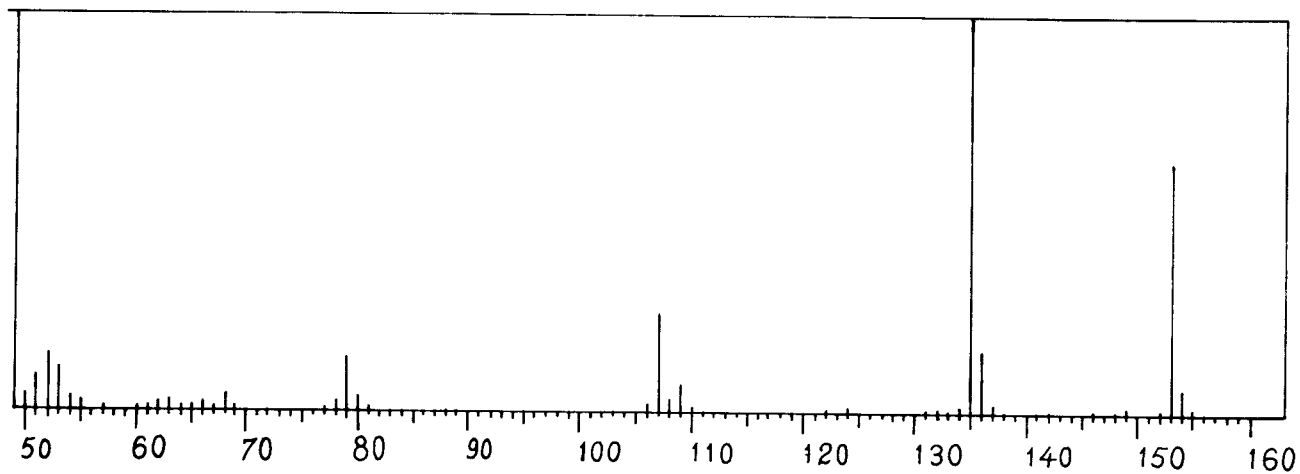
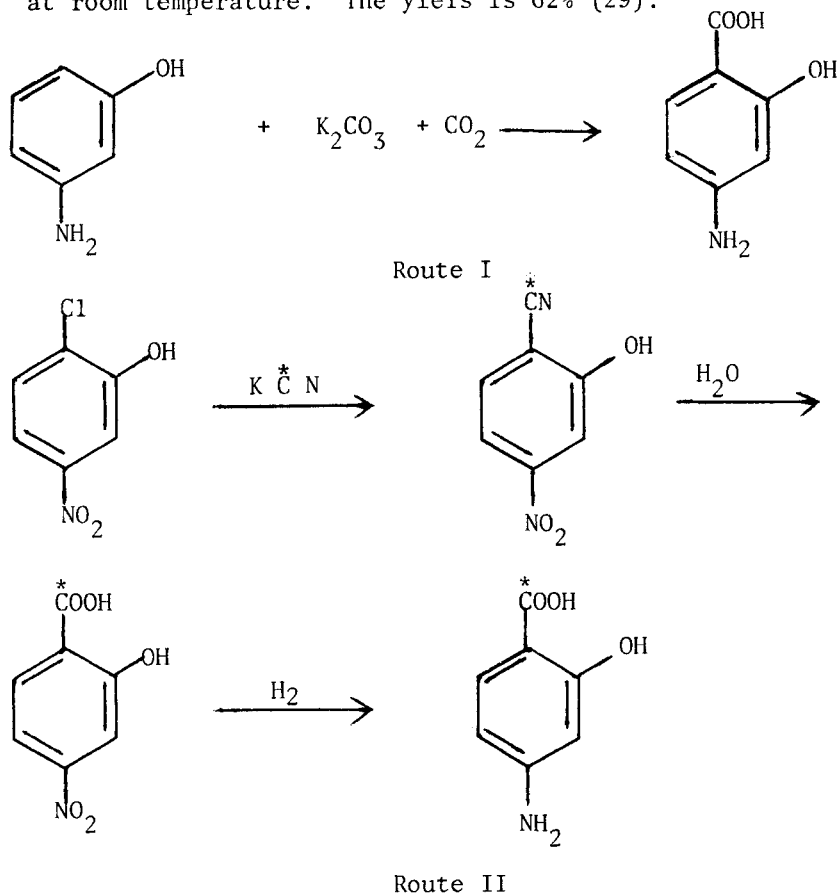


Fig. 10 : Mass Spectrum of p-Aminosalicylic acid.

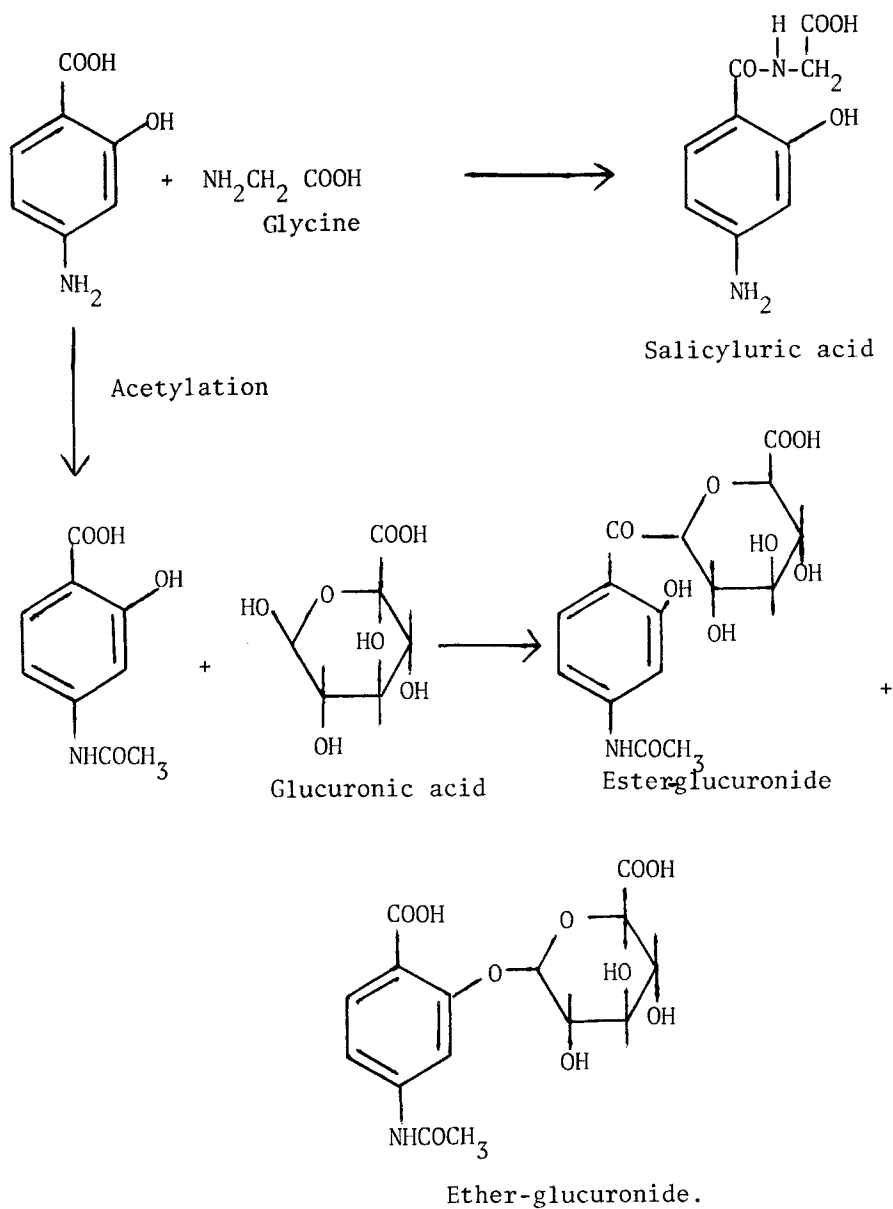
using potassium radio-cyanide to synthesise p-nitrosalicylic acid which was then reduced by Catalytic hydrogenation at room temperature. The yields is 62% (29).



4. Metabolism

The metabolism of PAS has been studied in both rabbits and humans. Bray et al (31) have studied in great detail the metabolism of PAS in the rabbit and found that approximately 50% of a dose of 1-2 gms is excreted unchanged and 50% as 4-acetamido-salicylic acid (m.p. 238-239°), which has been isolated and characterised. Also they have isolated 4-acetamido-salicylic acid from human urine after oral administration of 3 gms sodium 4-aminosalicylate. Considerable amounts were excreted unchanged.

This has also been proved by others (32). Zini (33) has studied the fate of 4-aminosalicylic acid in humans, the



Scheme I

urinary metabolites of PAS were acetylated-PAS, unchanged PAS, glycine-PAS and glucuronic acid-PAS conjugated compounds. Way et al (34), have reported the quantitative determination of the various metabolites of PAS excreted in the urine of human subjects by using countercurrent distribution and paper chromatography. They found that of the total dosage of PAS, 14 to 33% was excreted unchanged, 28 to 63% as acetyl-PAS, 0 to 26% as p-aminosalicyluric acid, 2 to 10% as unknown free amines and 3 to 10% as unknown bound amine. Lehman (35) have reported the occurrence of N-acetyl-PAS and N-(4-aminosalicyloyl) glycine in human blood plasma and urine after oral administration of PAS. He concluded that concomitant administration of high dosage of PAS with isoniazid probably depletes CO-A and thereby inhibits the acetylation of isoniazid. Wan et al., (36), have reported that the metabolism of PAS is mainly by acetylation which accounts for 50 to 70% of the absorbed dose and glycine conjugation to p-aminosalicyluric acid accounts for up to 25% of the dose. These two metabolites together constitute greater than 90% of the metabolites found in urine (37, 38). Metabolites of PAS are shown in scheme I.

5. Methods of Analysis

5.1 Non-aqueous titration

Kucharsky et al (39) and Chatten (40) have described a non-aqueous titration technique for the determination of PAS and Sodium PAS, both in pure form and in tablet formulation. Determination of pure PAS is based on titration of anhydrous acetone solution of the acid with 0.1N potassium hydroxide solution in anhydrous methanol using 0.5% of thymol blue solution in anhydrous methanol until the color of the indicator changes to blue. For tablet formulation the above determination is preceded by extraction of the specified amount of the tablet powder with anhydrous acetone.

For the determination of Sodium PAS the method is based on dissolving the specified amount of the substance on anhydrous methanol and titrated with 0.05N perchloric acid solution in dioxane using 0.5% thymol blue solution as indicator, until its color changes to peach. For the same in tablets the above determination is preceded by extraction of the specified amount of the powdered tablets with anhydrous methanol. These methods were reported to be specific even in the

presence of m-aminophenol (MAP). Butter and Ramsay (41) titrated PAS and its sodium salt potentiometrically with perchloric acid in glacial acetic acid and acetic acid. Carbon tetrachloride solvent mixture served as the titration medium.

Stockton and Zuckerman (42) determined sodium PAS and its solutions by potentiometric titration with perchloric acid in propylene glycol and isopropyl alcohol (1:1), using the same solvent mixture as the titration medium. The decomposition products MAP and sodium bicarbonate did not interfere. Das and Patel (43) employed the same titrant and solvent system.

Hunt and Blake (44) have described a non-aqueous titration method for the analysis of PAS and its salts and dosage forms. This method was reported to be specific in the presence of MAP. The method is based on titration with sodium methoxide in benzene-methanol using dimethylformamide as titration solvent. The end point is detected visually using thymol blue as indicator or potentiometrically. PAS and its decomposition product, m-aminophenol may be differentiated with this titration system. Salts of p-aminosalicylic acid are converted to the acid form by ion-exchanged chromatography prior to titration.

5.2 Diazometric Assay

USP XVIII method (45) for the determination of PAS, its salts and dosage forms, involves the diazotisation reaction and is based on procedures developed by Tarnoky and Bews (46) and Pesez (47,48).

Blake et al (49) have described a method for determination of sodium p-aminosalicylate in the presence of m-aminophenol. m-Aminophenol, the major breakdown product of p-aminosalicylic acid, if present, is also diazotised and constitutes an interference in the official assay procedure. In this method the PAS content and mixtures containing MAP is determined by the modification of the official assay procedure. The MAP is removed by passing the solution of the mixture in dimethylformamide through a column containing a strong cation exchange resin. The elute is then treated according to the official method.

5.3 Spectrophotometry

Coccia (50) has described determination of PAS, m-aminophenol and p-aminophenol colorimetrically by utilising their reaction with sodium nitropentacyanocobaltate to give an orange compound. The color produced obeys Beer's law at 440 nm in the range of 0 to 0.75 μg of PAS per ml. The compound obtained with PAS was prepared and its formula and molecular weight were obtained.

Rieder (51) has reported another colorimetric method for determination of free PAS in blood. The method is based on the coupling of PAS with diazotised sulphanilic acid in a strongly alkaline alcoholic medium. The resulting solution shows maximum extinction at 600 nm, but analysis were carried out at 630 nm in order to avoid interference. The color is stable for 30 minutes and the maximum error is $\pm 5\%$ in the range of 5 to 20 mg of PAS per 100 ml.

Another colorimetric method has also been reported (52) utilising reaction of PAS and MAP with ninhydrin solution.

5.4 Combined TLC and Colorimetry

Kinze (53) has reported the separation of PAS and MAP on layers of Alumina oxide by using ethanol or methanol as a developer. PAS remains on the base line in both instances. The spots are detected by spraying with 1% p-dimethylaminobenzaldehyde solution in ethanol treated with 5% hydrochloric acid. After extraction from the plate 2-60 mg of MAP can be determined colorimetrically at 420 nm with 1% furfuryl alcohol solution in anhydrous acetic acid.

5.5 Ultraviolet method

Moussa (54) has reported a U.V. method for determination of PAS in the presence of its degradation product MAP. The finely powdered tablets after extraction with ethanol is filtered and the filtrate is diluted and treated with borate buffer solution of pH 3 and the absorbance is measured at 300 nm against the buffer solution. There is no interference from MAP in amounts upto at least twice that of PAS.

PAS can be analysed spectrophotometrically by dissolving the sample in ethanol (95%) to give a concentration of about 15 $\mu\text{g/ml}$ and the absorbance of the solution so produced is measured at 303 nm. The $\log \xi$ values are given in Table 7 (16).

Table 7

<u>λ max nm</u>	<u>Log ξ</u>
235	2.765
274	3.622
303	3.624

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AZATHIOPRINE

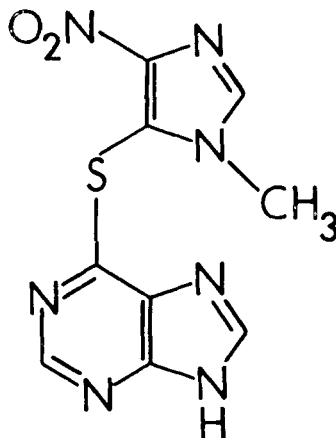
Wendy P. Wilson and Steven A. Benezra

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1. Description

1.1 Name, Formula, Molecular Weight

Azathioprine is 6-[(1-methyl-4-nitroimidazol-5-yl)thio]purine



$C_9H_7N_7O_2S$

277.3

1.2 Appearance, Color, Odor

Azathioprine is a pale yellow, odorless powder.

2. Physical Properties

2.1 Infrared Spectrum

The infrared spectrum of azathioprine is shown in Figure 1. The spectrum was obtained as a 0.4% dispersion of azathioprine in KBr with a Nicolet Model 7199 FT-IR spectrophotometer. The infrared assignments consistent with the structure of azathioprine are given in Table I.¹

Table I

Infrared Spectral Assignments for Azathioprine

<u>Band Frequency</u> <u>(Wavenumbers)</u>	<u>Structural Assignment</u>
921 and 857	C-H deformation attributable to the purine nucleus.
831 and 637	C-H deformation attributable to the imidazole ring.

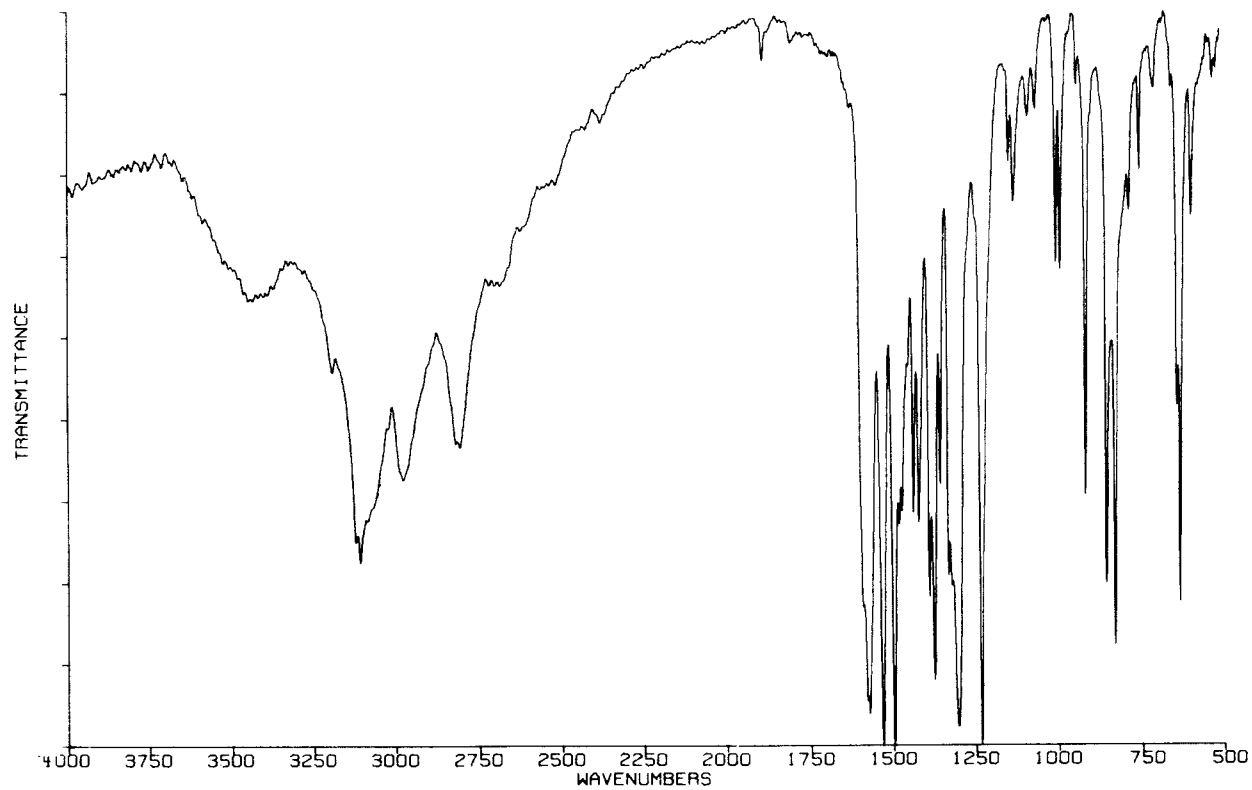


Figure 1 - Infrared Spectrum of Azathioprine

1233	C-N stretching from a tertiary amine and a purine nucleus.
1470 and 1390	C-H bending from a methyl group.
1537 and 1374	C-NO ₂ stretching (asymmetric and symmetric, respectively).
1595 and 1570	C=N stretching characteristic of the amidine groups in substituted purine and imidazole structures.
1893 and 1807	C-H deformation overtones attributable to the substituted purine and imidazole functions.
2810	C-H stretching indicative of a CH ₃ -N group.
2976	C-H stretching characteristic of a pyrimidine group.
3109	C-H stretching characteristic of imidazole groups.
3191	N-H stretching characteristic of a purine function.

2.2 Nuclear Magnetic Resonance (NMR) Spectra

The ¹H NMR spectrum of azathioprine is shown in Figure 2. The spectrum was obtained in deuterated dimethyl sulfoxide with a Varian XL-100A NMR spectrometer at 100 MHz. Chemical shifts referenced to DMSO at 2.51 ppm and consistent with the structure of azathioprine are presented in Table II.²

Table II

NMR Assignments for Azathioprine

<u>Proton</u>	<u>No. of Protons</u>	<u>Shift (ppm)</u>	<u>Multiplicity</u>
a	1	8.59	singlet
b	1	8.55	singlet
c	1	8.25	quartet
d	3	3.70	doublet
e	1	13.8	broad singlet

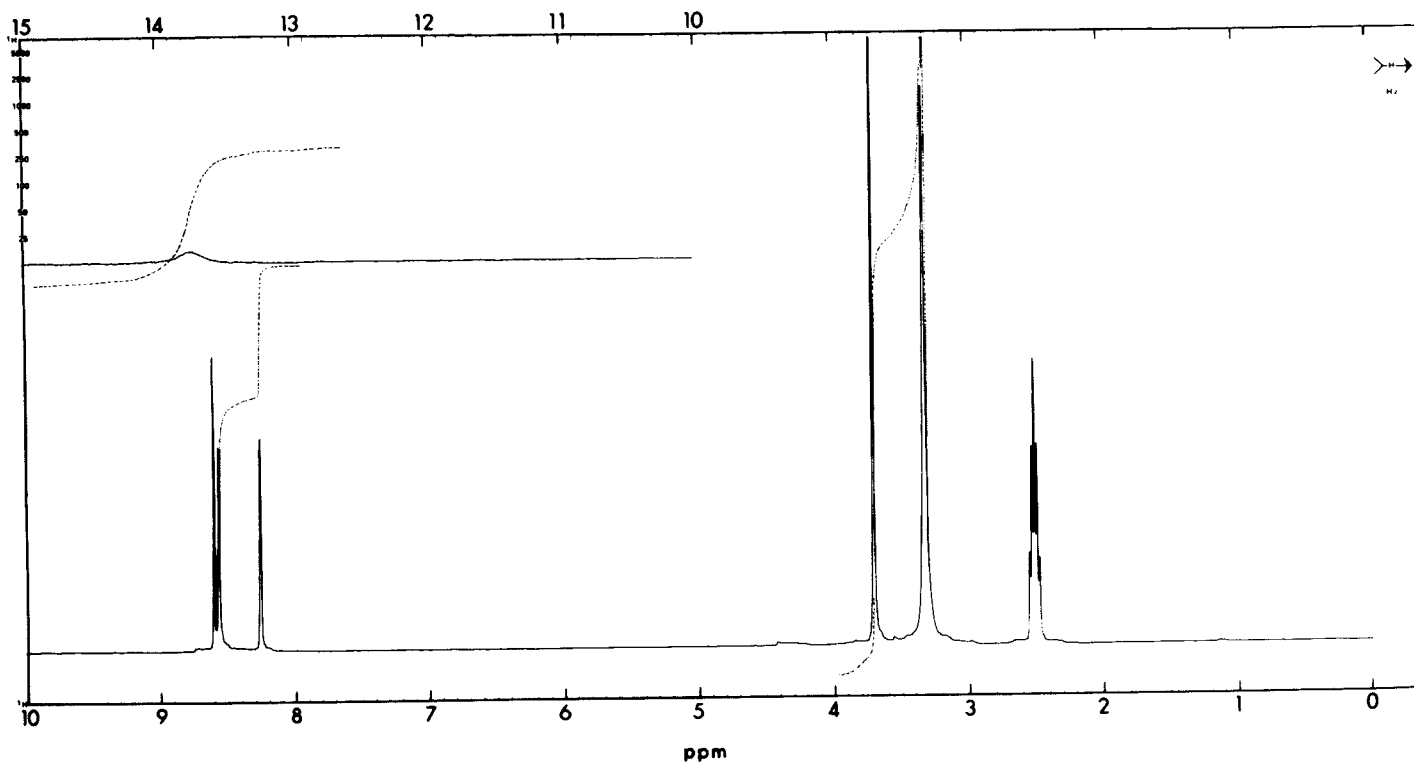
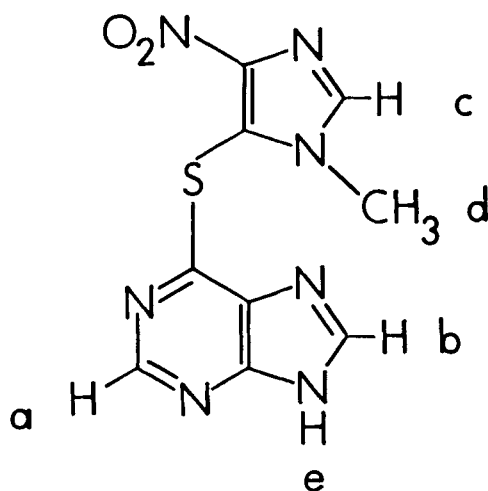


Figure 2 - ^1H Nuclear Magnetic Resonance Spectrum of Azathioprine



The ^{13}C NMR of azathioprine, shown in Figure 3, was obtained with a Varian CFT-20 NMR spectrometer at 80 MHz. Deuterated dimethyl sulfoxide was used as the solvent with tetramethylsilane as an internal standard. Carbon assignments for the ^{13}C NMR are given in Table III.³

Table III

<u>Carbon No.</u>	<u>Chemical Shift (ppm)</u>
2	151.6
4	150.6
5	130.0
6	154.6
8	144.5
2'	139.4
4'	149.7
5'	117.1
CH ₃	32.9

2.3 Ultraviolet (UV) Spectrum

The ultraviolet spectrum of azathioprine in methanol was obtained with a Beckman ACTA CIII UV spectrophotometer and is shown in Figure 4. Table IV gives UV data for azathioprine in various solvents.⁴

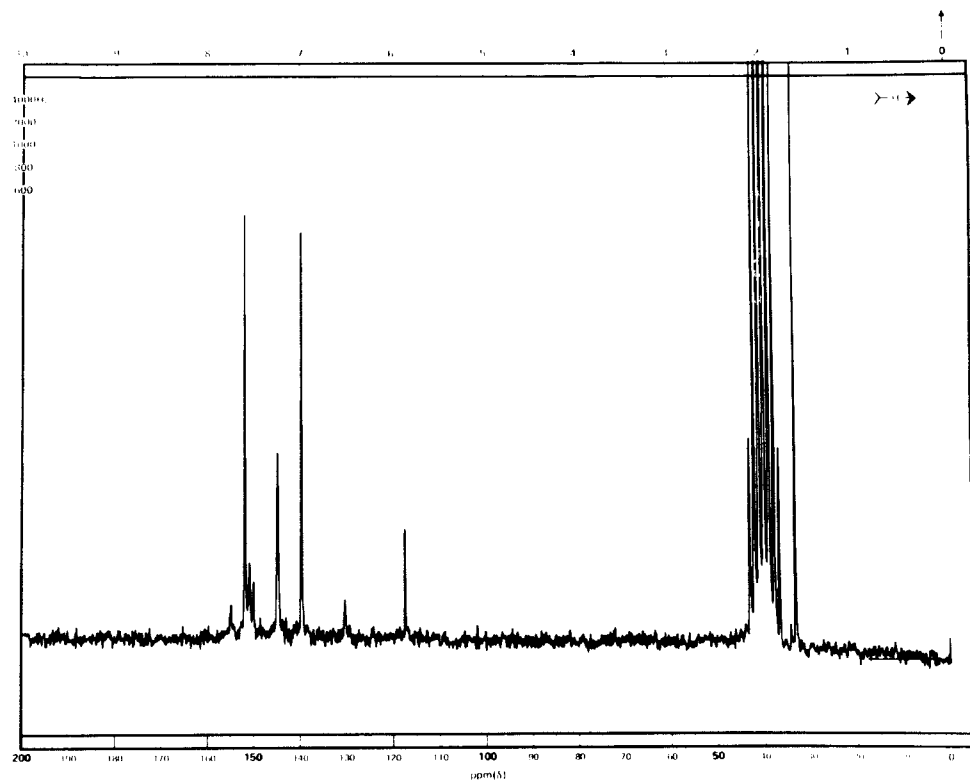


Figure 3 - ^{13}C Nuclear Magnetic Resonance Spectrum of Azathioprine

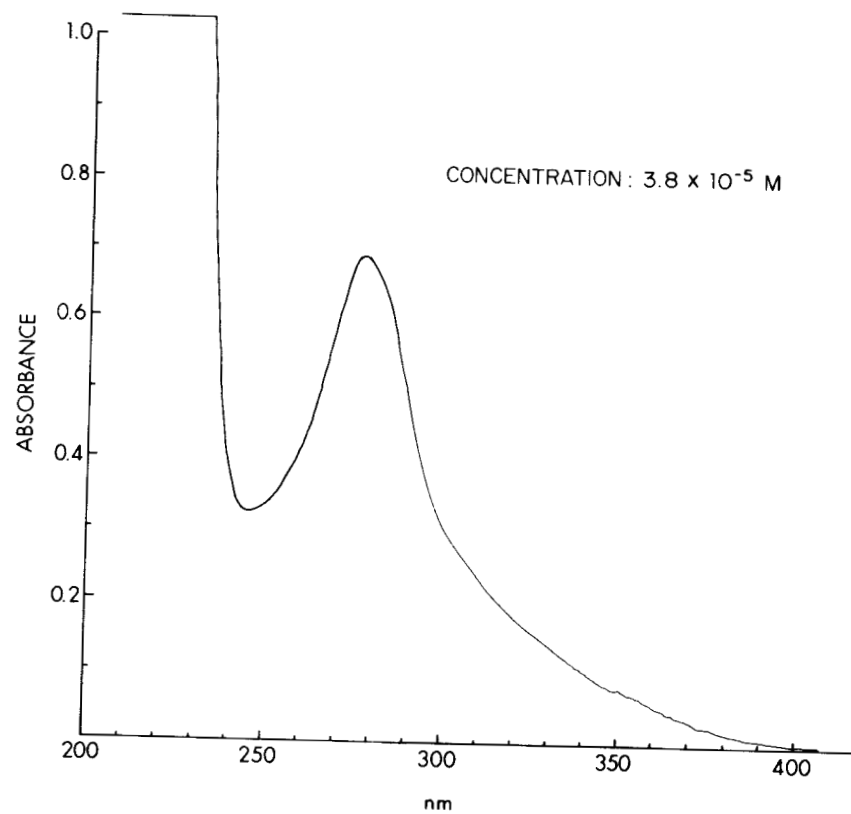


Figure 4 - Ultraviolet Spectrum of Azathioprine in Methanol

Table IV

UV Spectral Data for Azathioprine

Solvent	λ_{max} (nm)	ϵ_{max}
methanol	276	1.82×10^4
0.1N NaOH	285	1.55×10^4
0.1N HCl	280	1.73×10^4

2.4 Mass Spectra

The low resolution electron impact⁵ and field desorption⁶ mass spectra of azathioprine are shown in Figures 5 and 6.

The electron impact spectrum was obtained with a Varian MAT CH5-DF mass spectrometer. The sample was introduced into the ion source via direct probe at 285°C. The electron energy was 70 eV. The major fragment ions formed on electron impact are consistent with those found by Brent et al.⁷ Loss of NO₂ yields C₉H₇N₆S, m/z 231 (100%). Cleavage between sulfur and the purine ring with retention of charge on the purine ring results in (Pur)⁺, m/z 119 (42%). The fragment m/z 152 (10%) is formed by fission of the sulfur imidazole bond with rearrangement of a hydrogen to the purine moiety (PurS + H)⁺, and m/z 42 (45%) is C₂H₄N⁺.

The field desorption spectrum was obtained with a Varian MAT 731 mass spectrometer at an emitter heating current of 18 ma. The (M+1)⁺ ion (m/z 278), while absent from the electron impact spectrum, appears in the field desorption spectrum (4.6%). Other fragments present in the field desorption spectrum are m/z 231 (100%), M.-NO₂ and m/z 277.

2.5 Melting Point

Azathioprine melts and decomposes at approximately 240°C.⁸

2.6 Solubility

Azathioprine is very slightly soluble in water (~0.01% w/v at 25°).⁸ It is also slightly soluble in chloroform, ethanol and dilute mineral acids.⁸ Azathioprine is soluble in dilute solutions of alkali hydroxides with slow decomposition, dimethyl sulfoxide and poly-

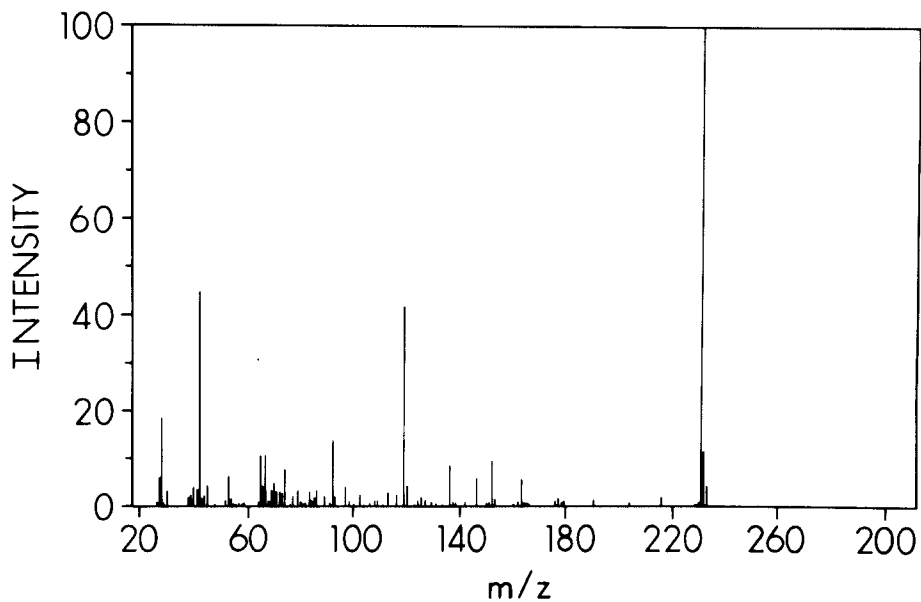


Figure 5 - Electron Impact Mass Spectrum of Azathioprine

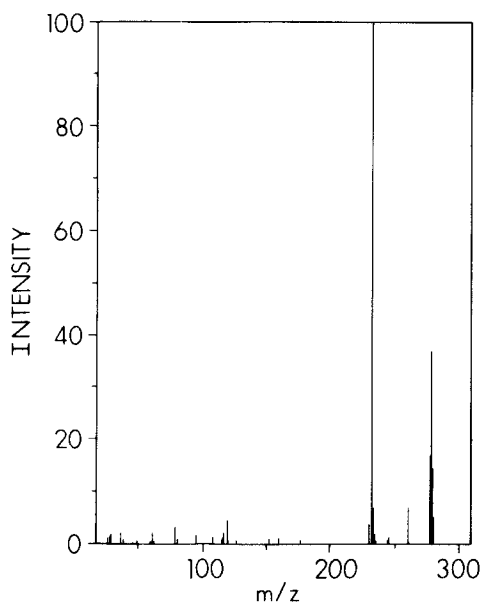


Figure 6 - Field Desorption Mass Spectrum of Azathioprine

ethylene glycol 400.^{8,9}

2.7 Dissociation Constant

The pK_{a2} of azathioprine is 8.2 at 25°C.⁸

3. Synthesis

Azathioprine is synthesized by the synthetic route shown in Figure 7. Diethylsuccinate (1) is reacted with methylamine (2) to give *N,N'*-dimethylsuccinamide (3) which in turn is reacted with $PCl_5/POCl_3$ to ring close to 1-methyl-5-chloroimidazole (4). The imidazole, 4, is converted to its salt with nitric acid to give 1-methyl-5-chloroimidazole nitrate (5). The imidazole nitrate, 5, is then converted to 1-methyl-4-nitro-5-chloroimidazole (6).

Ethyl cyanoacetate (7) is nitrosated, reduced and acetylated to give ethylacetamidocyanoacetate (8). The ring closure of 8 is done with formamide to give hypoxanthine (9) which is reacted with phosphorous pentasulfide to give 6-mercaptopurine (10). The imidazole (6) and 6-mercaptopurine (10) are condensed to yield azathioprine.¹⁰

4. Stability

Bulk samples of azathioprine are stable for at least two years at temperatures between 5°C and 37°C and one year at 50°C when stored in well closed, light resistant containers.^{11,12} The drug develops a dark orange surface after four weeks when exposed to fluorescent or ultraviolet light.¹¹

Azathioprine is stable in neutral and acidic solutions but is hydrolyzed to 6-mercaptopurine by alkali.^{13,14}

5. Metabolism and Pharmacokinetics

5.1 Metabolism

Azathioprine is initially split by glutathione in the liver to 6-mercaptopurine and 1-methyl-4-nitro-5-(S-glutathionyl)imidazole. To a much lesser extent azathioprine may be split between the purine ring and the sulfur to yield the metabolite 1-methyl-4-nitro-5-thio-

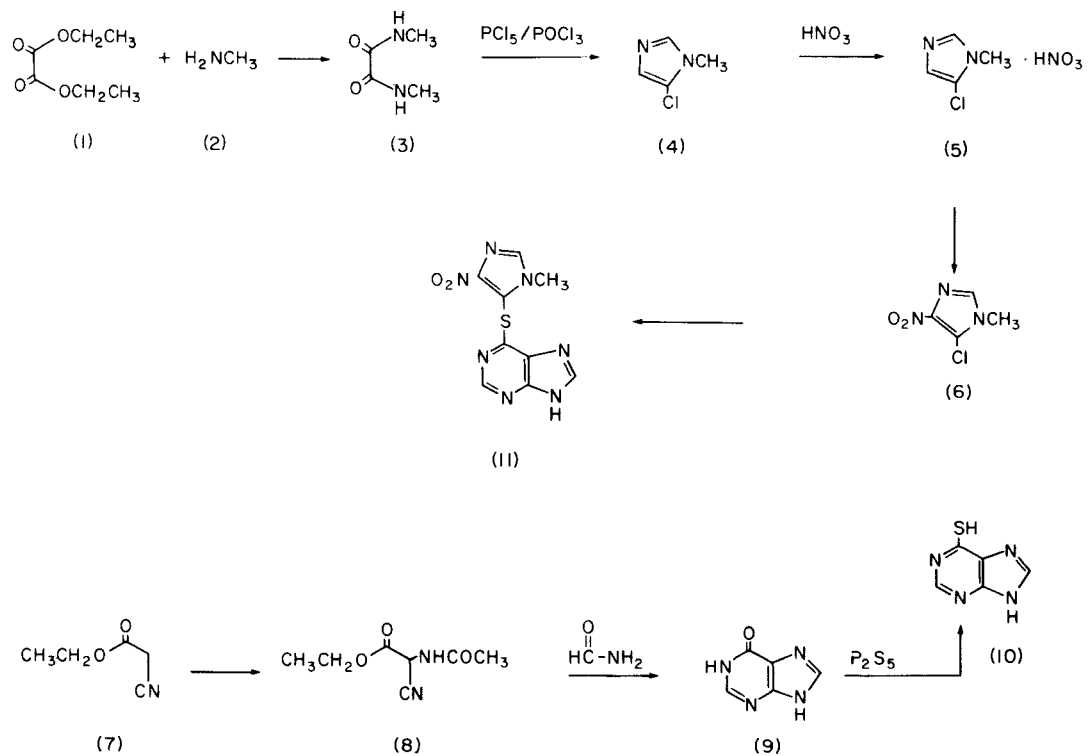


Figure 7 - Synthesis of Azathioprine

imidazole.¹⁵

The metabolism of the 6-mercaptapurine moiety follows two known pathways. It can be inactivated by xanthine oxidase to 6-thiouric acid or it can be converted to its active form, the ribonucleotide 6-thioinosinic acid, by hypoxanthine-guanine phosphoribosyl transferase in tissues.^{16,17,18}

The major urinary metabolite of the 1-methyl-4-nitro-5-(S-glutathionyl)imidazole moiety in man and in dogs is N,N'-[5-(methyl-4-nitro)imidazolyl]cysteine. The major metabolite in the rat, 1-methyl-4-nitro-5-(N-acetyl-S-cysteinyl)imidazole, accounted for only a small percentage of the dose in dogs and in man. Other metabolites of the methylnitroimidazole moiety include several 5-substituted amino-1-methyl-4-nitroimidazoles one of which, a glycine derivative, indicates that 6-mercaptapurine may also be displaced from azathioprine by nucleophilic attack of amino acids.^{19,20,21}

5.2 Excretion

In a human study using ³⁵S-azathioprine to follow the fate of the purine moiety, over 50% of the radioactive dose was excreted in the urine in twenty-four hours indicating a good absorption of the drug. Seventy percent of the ³⁵S had been excreted in forty-eight hours. Twelve percent unabsorbed material was excreted in the forty-eight hour stool specimens. Very little of the drug was eliminated unchanged. The major urinary metabolite was thiouric acid with less than 1% of the dose eliminated as 6-mercaptapurine and from 10% to 20% inorganic sulfate.^{15,22} Similar studies done in rats and in dogs gave similar results with the exceptions of relatively larger quantities of 6-mercaptapurine being excreted by rats and both rats and dogs excreted slightly more unchanged azathioprine.^{23,24}

Clearance of the methylnitroimidazole portion of the drug is much slower than that of the purine moiety. Following an oral dose of 90 mg of ¹⁴C-azathioprine the patient excreted only 20% of the ¹⁴C in the first twenty-four hours. In forty-eight hours only 37% of the ¹⁴C had been excreted in the urine in contrast to the 70% excretion of ³⁵S in forty-eight hours.^{15,21} Similar results were obtained in the rat and dog studies with ¹⁴C-azathioprine.^{19,20} Forty-two percent of the ¹⁴C had been excreted

ed by the dogs in 32 hours with very little radioactivity excreted after 32 hours.¹⁹

5.3 Tissue Distribution

The peak plasma radioactivity of the purine portion of azathioprine occurred at 2 hours in a patient treated with ³⁵S-azathioprine. The half-life of the plasma radioactivity was 4.5 to 5 hours and after 10 hours, when most of the remaining ³⁵S was inorganic sulfate, the clearance of radioactivity was much slower.¹⁵

Another patient was treated with ¹⁴C-azathioprine. Plasma radioactivity of the methylnitroimidazole moiety peaked at 4 hours at which time the plasma radioactivity was twice that found in the blood cells. After 12 hours the radioactivity had equilibrated between the plasma and the cells. At twelve hours the level of radioactivity was 40% of the peak value and this level persisted for 36 hours.¹⁵

The concentration of ³⁵S was determined in several organs of rats treated with ³⁵S-azathioprine. The highest concentration of ³⁵S was found in the liver 6 hours after administration of the drug. This concentration was five times that found in the blood plasma. Only traces of radioactivity were found in the fat-rich organs.²³ Another rat study showed that there is rapid hepatic extraction of azathioprine. After only 5 minutes a high proportion of the radioactive dose was recovered in the liver.²⁵

Radioactivity levels rapidly attained a maximum in the blood cells and then declined rapidly in dogs treated with ¹⁴C-azathioprine. The peak plasma radioactivity was reached about 5 hours after drug administration and after 8 hours the radioactivity had equilibrated between the plasma and the blood cells. The radioactivity then declined gradually over 48 hours.¹⁹

6. Methods of Analysis

6.1 Elemental Analysis

The elemental analysis of azathioprine is given in Table V.²⁶

Table VElemental Analysis of Azathioprine

<u>Element</u>	<u>Theory (%)</u>
C	38.98
H	2.55
N	35.36
O	11.54
S	11.57

6.2 Nonaqueous Titration

An accurately weighed sample of azathioprine is dissolved in dimethylformamide. The solution is titrated with standardized 0.1 N tetrabutylammonium hydroxide to the thymol blue endpoint. Precautions must be taken to prevent the absorption of atmospheric carbon dioxide. Each milliliter of 0.1 N tetrabutylammonium hydroxide is equivalent to 27.73 mg of azathioprine.⁹

6.3 Polarography

A differential pulse polarographic analysis is used to assay azathioprine tablets and azathioprine sodium for injection. The samples are dissolved, diluted with 0.1 N sulfuric acid and de-aerated with nitrogen. Using a dropping mercury electrode with a saturated calomel reference electrode, the polarogram is recorded from -0.60 volt to -1.00 volt. The height of the diffusion current is compared to that of a reference standard prepared in a similar manner to obtain the concentration of azathioprine in the formulations.⁹

6.4 Microbiological Assay

Harber and Maddocks described a method of estimating nanogram quantities of azathioprine by measuring the extent of growth inhibition of *Lactobacillus casei*. A modified folic acid assay medium containing between 20 and 200 ng azathioprine was inoculated with 2 drops of a stock solution of *Lactobacillus casei*. The cultures were incubated at 37°C for 18 hours and turbidity was then measured at 560 nm. A range of standards were similarly prepared and a standard curve was drawn from which the concentration of azathioprine was read.²⁷

6.5 Phosphorescence Spectroscopy

Azathioprine has been analyzed phosphorimetrically at -196°C . In alkaline ethanol, with excitation and phosphorescence wavelengths of 311 nm and 451 nm, respectively, azathioprine had a detection limit of 2.6 $\mu\text{g/ml}$ and the concentration to phosphorescence relationship was linear over at least two orders of magnitude of concentration. Phosphorescence in neutral ethanol was observed at 442 nm with an excitation wavelength of 300 nm. The detection limit of azathioprine under these conditions was 10 $\mu\text{g/ml}$.²⁸

6.6 Fluorimetric Analysis

Azathioprine and its metabolite 6-mercaptopurine have been successfully quantitated in plasma using a fluorimetric assay. The 6-mercaptopurine was first derivatized with phenyl mercuric acetate. This derivative could then be extracted from the plasma with toluene. The derivative was converted back to 6-mercaptopurine with 0.1 N hydrochloric acid and the toluene was removed. The 6-mercaptopurine was then oxidized to purine 6-sulfonate with potassium chromate followed by sodium metabisulfate and sodium hydroxide solutions. The fluorescence of the solution was measured at 398 nm with an excitation wavelength of 288 nm.

Azathioprine was hydrolyzed to 6-mercaptopurine with 5 N sodium hydroxide. After neutralization with 5 N hydrochloric acid the derivatization, extraction, oxidation and fluorimetric analysis steps were followed as for the 6-mercaptopurine. The concentration of azathioprine was calculated from the difference in the 6-mercaptopurine concentration in the hydrolyzed and non-hydrolyzed samples.²⁹

6.7 Chromatography

6.7.1 Column Chromatography

Nelson and coworkers have separated several azathioprine metabolites by column chromatography on DEAE-Sephadex columns. The metabolites were eluted with pH 4.7 triethylammonium acetate buffer. 10 mM β -mercaptoethanol was added to the mobile phase to prevent oxidation of the thiopurines. Azathioprine was converted to 6-mercaptopurine on the column under these conditions and could not

be separated. Detection was UV at 254 nm.³⁰

Measurement of azathioprine, 6-mercaptopurine and 6-thiouric acid in urine was achieved on the cation exchange resin Zeo Karb 225. 6-Thiouric acid was eluted first with 30-40 ml water which was then evaporated to dryness. 6-Mercaptopurine was eluted next with 15 ml of 20% ammonium hydroxide and then evaporated to dryness. Azathioprine was converted to 6-mercaptopurine by the addition of glutathione to the pH 8.9 adjusted urine, which was then chromatographed as described above. The eluates were dissolved in 5% perchloric acid and concentrations were determined by the decrease in extinction measured after the addition of mercuric chloride. 6-Thiouric acid was measured at 345 nm and 6-mercaptopurine was measured at 330 nm. The concentration of azathioprine was determined by the difference in 6-mercaptopurine concentration before and after the addition of glutathione.³¹

Azathioprine has been separated from other purines on Sephadex G-10. The mobile phase was 0.05M, pH 7 phosphate buffer. The recovery of the chromatographed purines was quantitative.³²

6.72 High Performance Liquid Chromatography (HPLC)

Table VI gives various HPLC systems used for azathioprine and its metabolites.

6.73 Paper Chromatography

Azathioprine and several methylnitroimidazole metabolites have been separated on Whatman no. 3 filter paper. The two solvent systems used were n-butanol:acetic acid:water (4:1:5), the top layer was used, and n-propanol:water (7:3). The chromatograms were developed for 20 hours and the compounds were detected under ultraviolet light. The R_f values for azathioprine were 0.75 in the n-butanol system and 0.86 in the n-propanol system.^{19,20}

6.74 Thin Layer Chromatography (TLC)

TLC systems used for azathioprine and its metabolites are given in Table VII.

Ito and Fujita describe the use of 3,5-di-tert-butyl-1,2-benzoquinone-iron (III) chloride as a TLC spray reagent for the detection of thiols. Fifty nanomoles of

Table VI

HPLC Systems for Azathioprine

<u>Column</u>	<u>Mobile Phase</u>	<u>Flow (ml/min)</u>	<u>Retention Time (min)</u>	<u>Detection</u>	<u>Ref</u>
PA-38 pellicular anion-exchange resin (3m x 1mm i.d.)	gradient from 0.03 M to 4.0 M ammonium acetate pH 4.7	0.4	AZA- 18 6-MP- 4 6-TU- 78	254 nm or 350 nm	30
μBondapak C ₁₈ (30cm x 3.9mm i.d.)	11% Acetonitrile in 0.01 M sodium acetate buffer pH 4.0	2	AZA- 8	280 nm	33
5μ ODS-Hypersil (10cm x 5mm i.d.)	Methanol:25 mM potassium dihydrogen phosphate : glacial acetic acid (20:79.5:0.5) pH 4.50	1.5	AZA- 4 6-MP- 2 MNTI- 2.5 MNHI- 1	240 nm	34
μBondapack C ₁₈ (30cm x 4mm i.d.)	Water : Methanol (70:30)	2	AZA- 3.2	280 nm	35
μBondapak C ₁₈	Acetonitrile:water: glacial acetic acid (15:85:0.02)		AZA- 11	280 nm	36

Table VI continued

<u>Column</u>	<u>Mobile Phase</u>	<u>Flow (ml/min)</u>	<u>Retention Time (min)</u>	<u>Detection</u>	<u>Ref</u>
Aminex-27 (100cm x 1.24mm i.d.)	gradient from 0.015 M to 6.0 M sodium acetate pH 4.0	0.13	AZA- 300 N,N'-MNIC- 925 MNTI- 1020	254 nm or 280 nm	19
PA-38 pellicular anion exchange resin (3m x 1mm id)	0.02 M ammonium formate pH 4.9	0.4	AZA- 15 6-MP- 7	280 nm	20

AZA	azathioprine
6-MP	6-mercaptopurine
MNHI	1-methyl-4-nitro-5-hydroxyimidazole
MNTI	1-methyl-4-nitro-5-thioimidazole
N,N'-MNIC	N,N'-[5-(methyl-4-nitro)imidazolyl)cysteine
6-TU	6-thiouric acid

Table VII

TLC Systems for Azathioprine

<u>Adsorbent</u>	<u>Mobile Phase</u>	<u>R_f</u>	<u>Detection and Comments</u>	<u>Ref</u>
Silica gel 60 F 254	acetic acid:ethanol (1:9)	6-MP- 0.59	Azathioprine metabolites were converted to phenyl mercury derivatives before chromatography. Following conversion back to the parent thiols by spraying with 2 N HCl, compounds were detected by low temperature (-196°C) phosphorescence at 254 nm and 366 nm. Detection of mercury can also be achieved by spraying chromatogram with 0.1 N acetic acid followed by a dithiozone solution.	39
		6-TU- 0.30		
	ammonia:butanol: water (1:60:39)	6-MP- 0.63		
		6-TU- 0.37		
	heptane:chloroform: ethanol (1:1:1)	6-MP- 0.58		
		6-TU- 0.04		
Cellulose	0.1 M hydrochloric acid	AZA- 0.66	Low temperature (-196°C) phosphorescence detection was used with excitation and phosphorescence wavelengths of 342 nm and 485 nm respectively and 320 nm and 448 nm respectively.	28
		6-MP- 0.44		

Table VII continued

<u>Adsorbent</u>	<u>Mobile Phase</u>	<u>R_f</u>	<u>Detection and Comments</u>	<u>Ref</u>
Cellulose	0.1 N hydrochloric acid	AZA- 0.70 6-MP- 0.43 6-TU- 0.24	low temperature (-196°C) luminescence detection at 366 nm	37
	water	AZA- 0.70 6-MP- 0.26 6-TU- 0.75		
	isopropanol:methanol: water:ammonia (60:20:20:1)	AZA- 0.87 6-TU- 0.25 6-MP- 0.55		
ECTEOA- cellulose	acetone:0.1 M sulfuric acid:ethyl acetate (45:10:45)	AZA- ~0.8 6-MP- 0.45	Viewed under an UV lamp. 6-Mercaptopurine fluoresced at 254 nm and 366 nm. Azathioprine quenched fluorescence at the same wavelengths.	38
	acetone:water (20:80)	AZA- 0.47 6-MP- 0.36		

AZA azathioprine
6- MP 6-mercaptopurine
6- TU 6-thiouric acid

6-mercaptopurine was detected on a cellulose TLC plate with this reagent.⁴⁰

6.75 Thin Layer Electrophoresis

The separation of azathioprine from other thio-purine derivatives has been achieved on both silica gel and ECTEOLA-cellulose thin layer chromatography plates with the use of low-voltage thin layer electrophoresis. A 0.7% triethanolamine buffer adjusted to pH 9.5 with acetic acid was used with the silica gel plates and a 5% pyridine buffer adjusted to pH 6.0 with acetic acid was used with the ECTEOLA-cellulose. The electrophoresis was carried out at 300 V at 4°C for 3 hours for the silica gel plates and 3.5 hours for the ECTEOLA-cellulose plates. After electrophoresis the plates were air dried then dipped into an ammonia fume chamber for 30 seconds. Low temperature (-196°C) phosphorescence detection was performed at 254 nm and 366 nm.⁴¹

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BENZYL BENZOATE

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and
Jaber S. Mossa

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1. Description

1.1 Nomenclature

1.11 Chemical Names

- a) Benzoic acid phenylmethyl ester.
- b) Benzoic acid benzyl ester.
- c) Benzylbenzenecarboxylate.

1.12 Generic Name

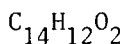
Benzyl **benzoate**.

1.13 Trade Names

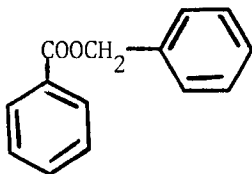
Ascarbin; Ascabiol; Benylate; Vanzoate; Venzonate.

1.2 Formulae

1.21 Emperical



1.22 Structural



1.23 CAS No.

120-51-4

1.24 Wiswesser Line Notation

RVOIR.

1.3 Molecular Weight

212.25

1.4 Elemental Composition

C, 79.22%; H, 5.70%; O, 15.8%.

1.5 Appearance, Color, Taste, Odor

Leaflets or colorless oily liquid, faint, pleasant aromatic odour, sharp burning taste.

2. Physical Properties

2.1 Boiling Range

323 - 324°C (1), (2).

bp₁₆ 189 - 191°C (2); bp₁₁ 170°C (1)

bp_{4,5} 156°C (2).

2.2 Melting Point

21°C.

2.3 Density

d₄²⁵ 1.118 (2), d₄²⁵ 1.1121 (1).

2.4 Refractive Index

n_D²¹ 1.5681 (2)

n_D²⁰ 1.5680 (1)

n_D²⁰ 1.568 - 1.570 (3).

2.5 Solubility

Insoluble in water or glycerol, miscible with alcohol (95%), chloroform, ether, oils, acetone, benzene, methanol, petroleum ether (1-3).

2.6 Identification

Boil 2 g with 25 ml of alcoholic potassium hydroxide solution for 2 hours under a reflux condenser. Remove the alcohol on a water-bath, add 50 ml of water, and distill until the liquid distilling is no longer turbid.

The liquid remaining in the flask, after acidification with dilute hydrochloric acid, yields a white crystalline precipitate of benzoic acid.

To the distillate add 2.5 g of potassium permanganate and 2 ml of sodium hydroxide solution, boil for 15 minutes under a reflux condenser, cool, and filter. The filtrate after acidification with dilute hydrochloric acid, yields a white crystalline precipitate of benzoic acid (3).

2.7 Spectral Properties

2.71 Infrared Spectrum

The infrared spectrum of benzylbenzoate is recorded as a film on a Unicam SP 3-300 Spectrophotometer and is shown in Fig. 1. The assignments for the characteristic bands in the infrared spectrum are listed in Table 1.

Table 1

IR Characteristics of Benzylbenzoate

Frequency CM^{-1}	Assignment
1720	C = O (ester)
1601	C = C aromatic
1590	C = C aromatic
1275	C - O - C
1110	C - O - C
710, 700	Aromatic monosubstitution.

Other finger print bands characteristic of benzylbenzoate are 3060, 3030, 1500, 1450, 1380, 1315, 1180, 1070, 1025 and 740. The IR spectral data have also been reported (1,4).

2.72 Ultraviolet Spectrum (UV)

The UV spectrum of benzylbenzoate in ethanol was scanned from 400-200 nm using Varian Cary 219, six maxima and six minima were observed (Fig.2). The maxima were located at 229, 256, 263, 266, 272 and 280 nm. The minima occur at 215, 254, 260, 265, 270 and 277.

The UV spectral data of benzylbenzoate have also been reported (1,5). The $E_{1\%}^{1\text{cm}}$ = 843 at 230 nm (6).

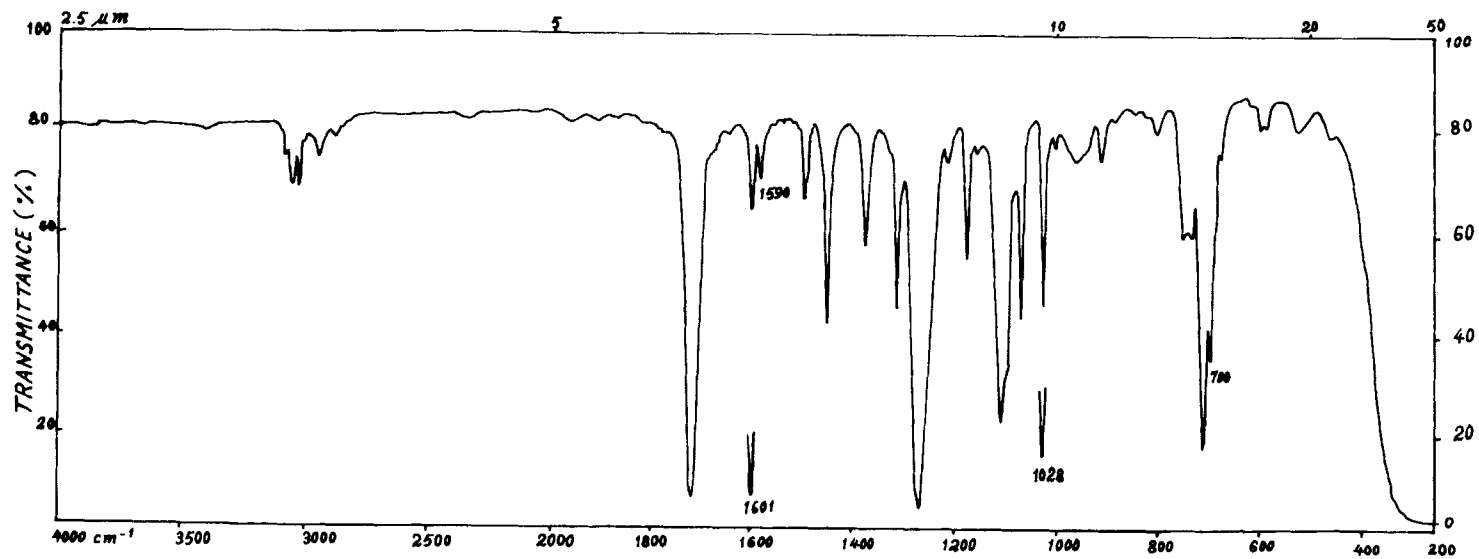


Fig.1 : Infrared spectrum of benzyl benzoate in nujol.

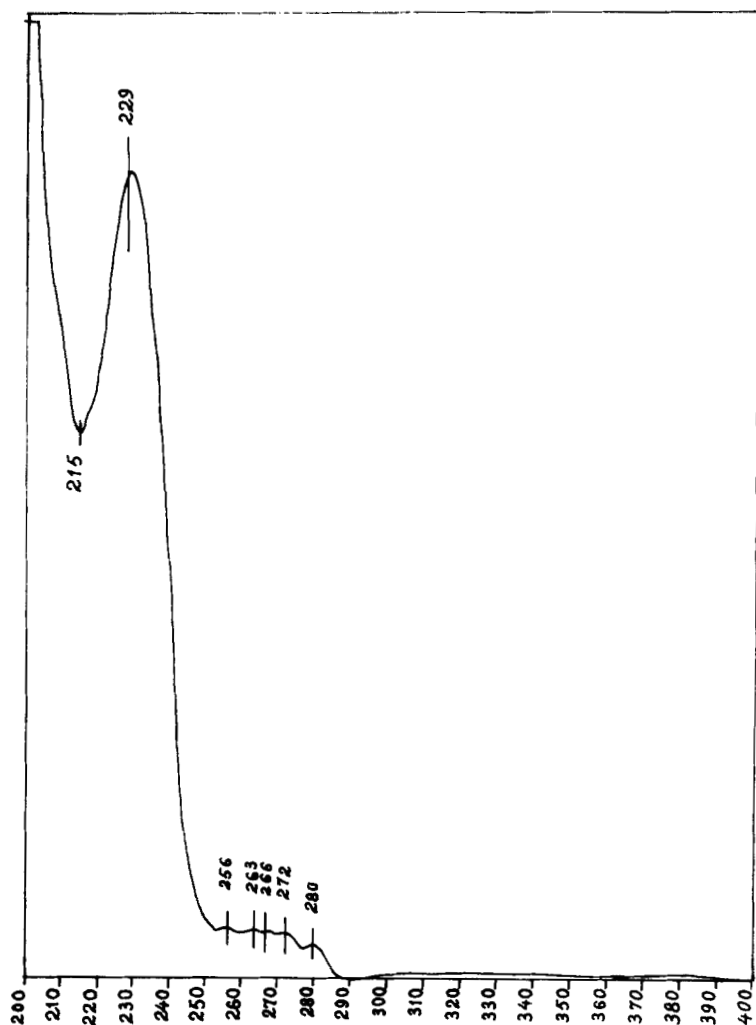


Fig. 2. Ultraviolet spectrum of benzyl benzoate in ethanol.

2.73 Nuclear Magnetic Resonance Spectrum

2.731 Proton Spectrum

The proton NMR spectra of benzylbenzoate in deuterated chloroform and in DMSO-D6 are shown in Fig. 3a and Fib. 3b. These were recorded on a Varian T-60A, 60 MHz NMR spectrometer, using tetramethylsilane as an internal reference. The PMR spectra assignment of benzylbenzoate are given in Table 2.

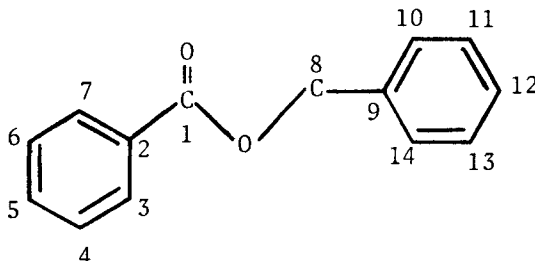
Table 2
PMR Characteristics of Benzylbenzoate

Protons	Chemical shifts	
	CDCl ₃	DMSO-D ₆
-CH ₂	5.26	5.39
-2H, 6H (adjacent to C)	7.23	7.47
Other aromatic protons.	8.00	8.03

Other PMR spectral data have also been reported (7,8).

2.732 ¹³C Spectrum

¹³C NMR spectrum of benzylbenzoate in carbon tetrachloride using tetramethylsilane as an internal standard was recorded using Jeol FX 100 MHz instrument at ambient temperature and using 10 mm sample tube. The data consist of 8192 data points over a 5000 Hz spectral width Fig. 4. The carbon chemical shift values are shown in Table 3. (9-11).



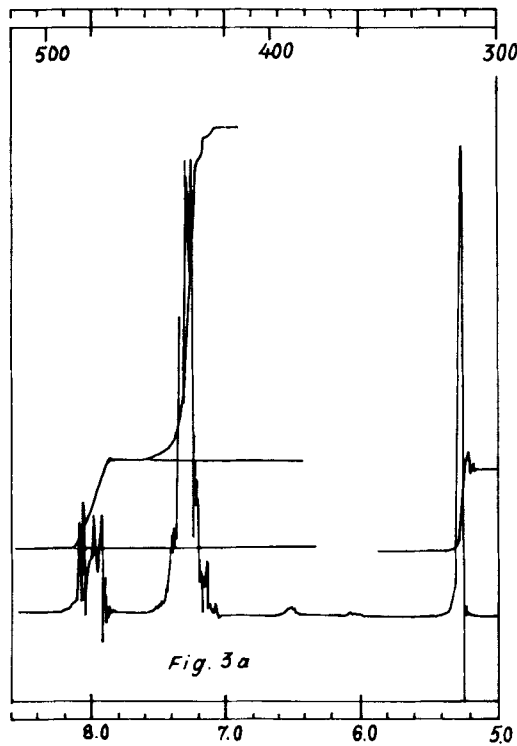


Fig. 3a. PMR spectrum of benzyl benzoate and TMS in CDCl_3 .

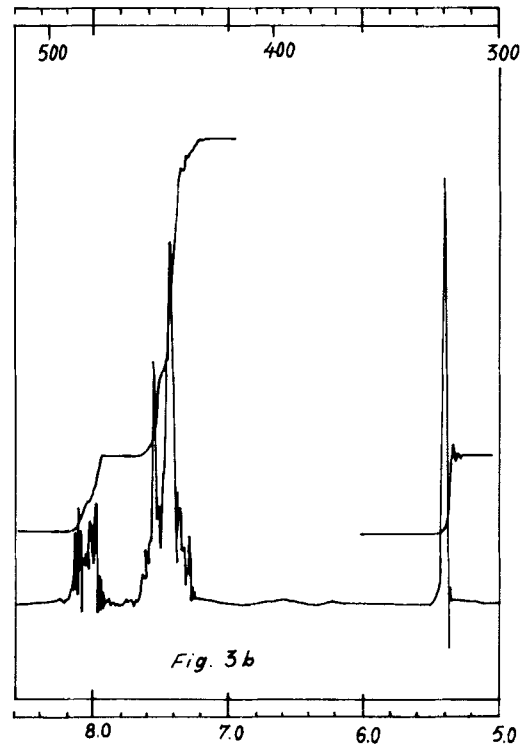


Fig. 3b. PMR spectrum of benzyl benzoate and TMS in DMSO-D_6 .

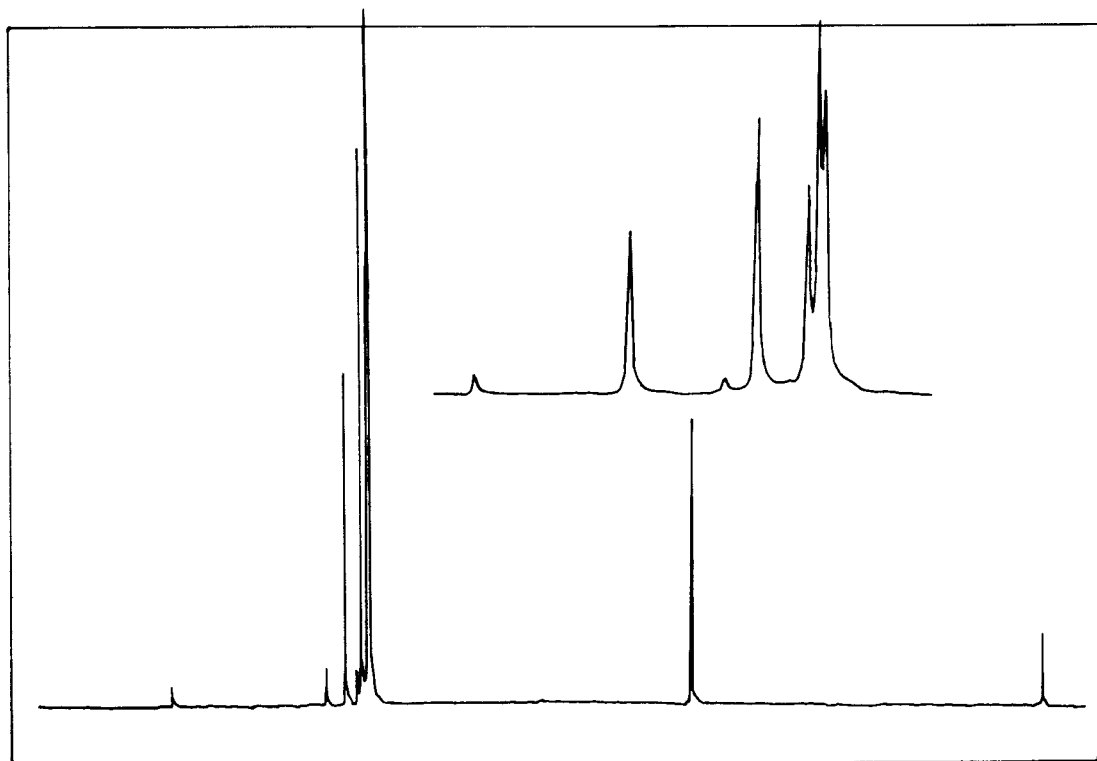


Fig. 4: ^{13}C NMR spectrum of benzyl benzoate in CCl_4

Table 3

 ^{13}C NMR Characteristics of Benzylbenzoate

Carbon No.	Chemical Shift.	Carbon No.	Chemical Shift.
1	165.63	8	66.40
2	130.30	9	136.29
3	129.56	10	128.40
4	128.20	11	128.01
5	132.59	12	128.01
6	128.20	13	129.01
7	129.56	14	128.40

2.74 GC/Mass Spectrum

The GC/Mass spectrum was recorded on Ribermag R 10-10 GC/Mass spectrometer using 3% SE 30, packed glass column. The GC trace shows a retention time of 6.57 minutes. The mass spectrum was obtained by conventional electron impact ionisation at 70 eV, shows a molecular ion M^+ at m/e 212 and shown in Fig. 5. Other prominent fragments and their relative intensity are shown in Table 4.

Table 4

m/e	Relative Intensity	Fragment
77	100.00	C_6H_5^+
91	77.3	$\text{C}_6\text{H}_5\text{CH}_2^+$
105	95.9	$\text{C}_6\text{H}_5\text{CO}^+$
65	42.9	
51	57.4	

The mass spectrum of benzyl benzoate has also been reported (1, 9).

3. Synthesis

Three main methods are used for preparation of benzyl benzoate.

BB1 SCAN 156 SIGMA=S RT=6:57 BACKGD= 15 X 100 100%=4046848
TITLE: SAMPLE BENZYL BENZOATE: 150-230 DEG(10 DEG/ MIN); 3%SE30; E.I.

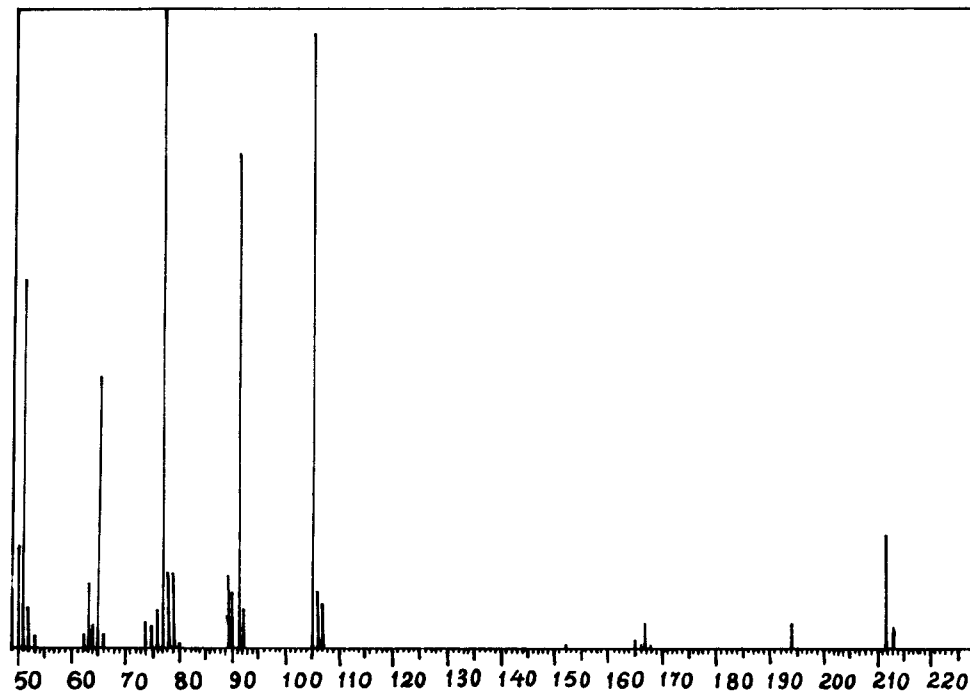
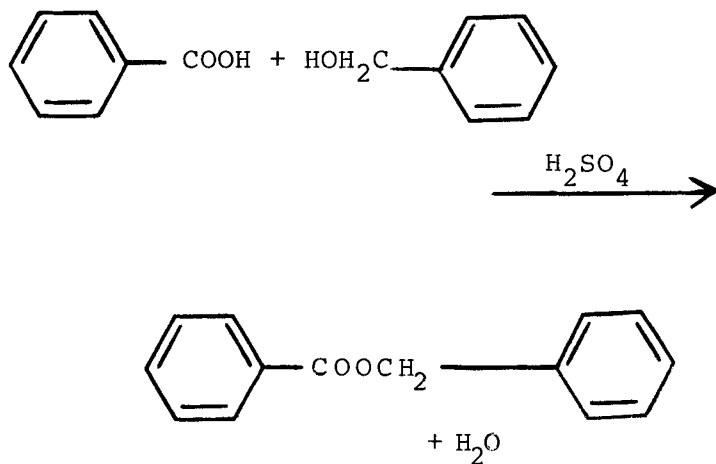
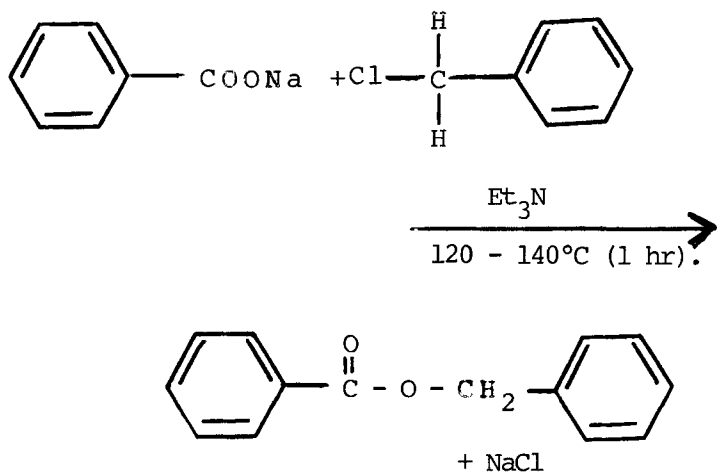
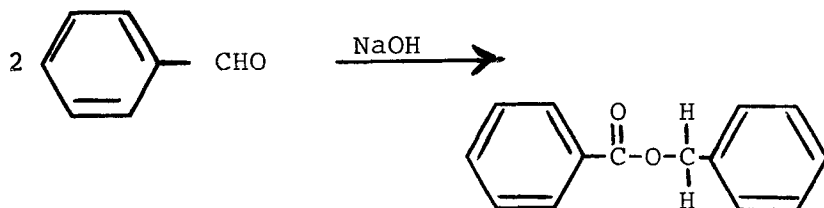


Figure 5. Mass spectrum of benzyl benzoate.

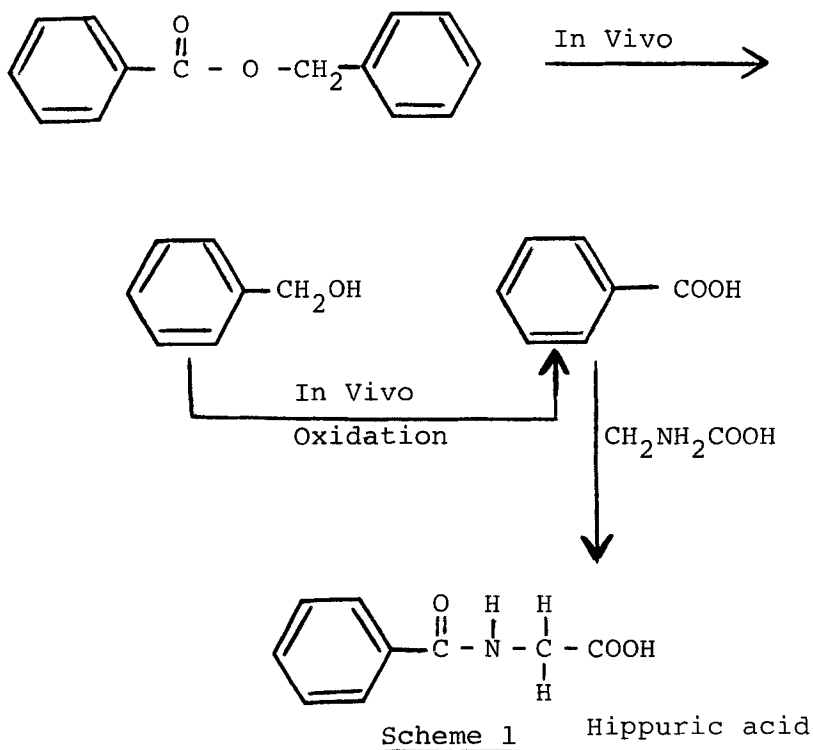
I) Estrification of benzoic acid with benzylalcohol(12,13)II) Transposition between sodium benzoate and benzyl-chloride. (12, 13).

III) Condensation of two molecules of benzaldehyde in the presence of sodium hydroxide (13).



4. Metabolism

Benzylbenzoate is rapidly hydrolysed in vivo to benzoic acid and benzylalcohol. Benzylalcohol in turn is oxidised to benzoic acid which is then conjugated with glycine to form hippuric acid (Scheme 1)., (5,6).



5. Methods of Analysis

5.1 Titrimetric Method

The U.S.P. XVIII (14) describes a titrimetric method for determination of benzylbenzoate. The method is based on the hydrolysis of a weighed amount of the ester with a known volume of 0.5N alcoholic potassium hydroxide by boiling under reflux for an hour. Then the reaction mixture is cooled, phenolphthalin T.S. as indicator is added and the excess alkali is back titrated with 0.5N Hydrochloric acid. A blank determination is also performed. 1 ml of 0.5N alcoholic potassium hydroxide \equiv 106 mg of benzylbenzoate ($C_{14}H_{12}O_2$).

5.2 Spectrophotometric Methods

Quantitative determination of benzylbenzoate as pure drug and in benzylbenzoate lotion by a spectrophotometric methods have been reported (15,16). The methods involve heating the sample under reflux with 10% alcoholic potassium hydroxide for 5 minutes and measuring the extinction of the cooled reaction mixture after dilution with water or ethanol at 268 nm. Beer's law is obeyed for up to 250 $g\ ml^{-1}$ of hydrolysed benzylbenzoate. Interference from other ingredients of the sample (e.g., oleic acid and triethanolamine) is negligible.

5.3 Spectrotitrimetric Methods

Benzylbenzoate and dibutylphthalate are determined in mixtures by measurement of the absorbancy at 250 nm and the quantity of alkali required to saponify the esters (17). The concentration of the esters are calculated by application of a differential equation for which the absorptivity and the saponification contents of the components are required. Analysis of five known mixtures showed average recoveries of 100.8% for benzylbenzoate and 99.97% for dibutylphthalate. Application to cloth patches impregnated with insect repellents containing these esters gave average recoveries of 101.4% for benzylbenzoate and 99.66% for dibutylphthalate.

5.4 Gas Chromatographic Method

A gas-chromatographic method has been described for the determination of benzylbenzoate as a product of catalytic oxidation of toluene (18). The determination was

carried out on a column (2 m) of 20% of carbowax 20 M on chromosorb W (60 to 80 mesh) operated at 200° with N (44 ml min⁻¹) as carrier gas, flame ionisation detection and acetophenone or benzylalcohol as internal standard.

5.5 Proton Magnetic Resonance Method

An accurate, simple and precise PMR procedure has been developed in our laboratory for the quantitation of benzylbenzoate and benzylcinnamate as pure drugs and in Peru and Tolu balsams (19). The method is based on the integration of the benzylmethylene protons of benzylbenzoate appearing at 5.30 ppm (Fig. 6). In Peru and Tolu balsam the corresponding peak appears at 5.32 ppm (Figs. 7 & 8).

Ethylbenzoate is chosen as the internal standard, since it has methylene protons that provide comparable area of integration. Acetone, rather than acetone-D₆, is employed as the solvent, since it is inexpensive and dissolves all balsam constituents as well as the internal standard.

The average recovery of pure benzylbenzoate in standard mixture is 100.2 ± 0.38 w/w. This method also offers the advantage of individually quantitating the esters, rather than the total ester contents in the medicinally used balsams. Moreover the spectrum of the balsam provides a useful mean for estimating the exact ratio of benzylbenzoate and benzylcinnamate, by simply measuring their corresponding benzylmethylene protons integrals. Also the PMR spectra of the esters and balsams are specific means of identification.

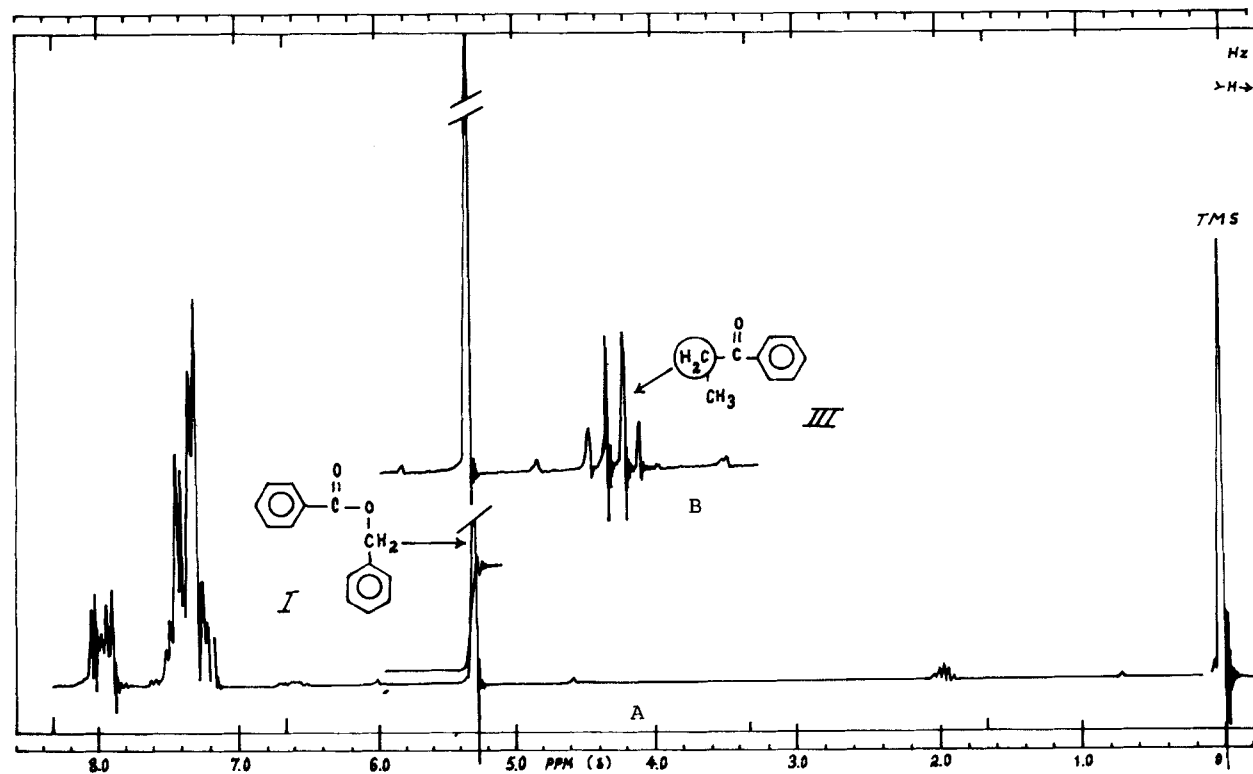


Fig. 6: A. PMR spectrum of benzyl benzoate in acetone-D₆.
 B. Part of the PMR spectrum of benzyl benzoate and ethyl benzoate in acetone.

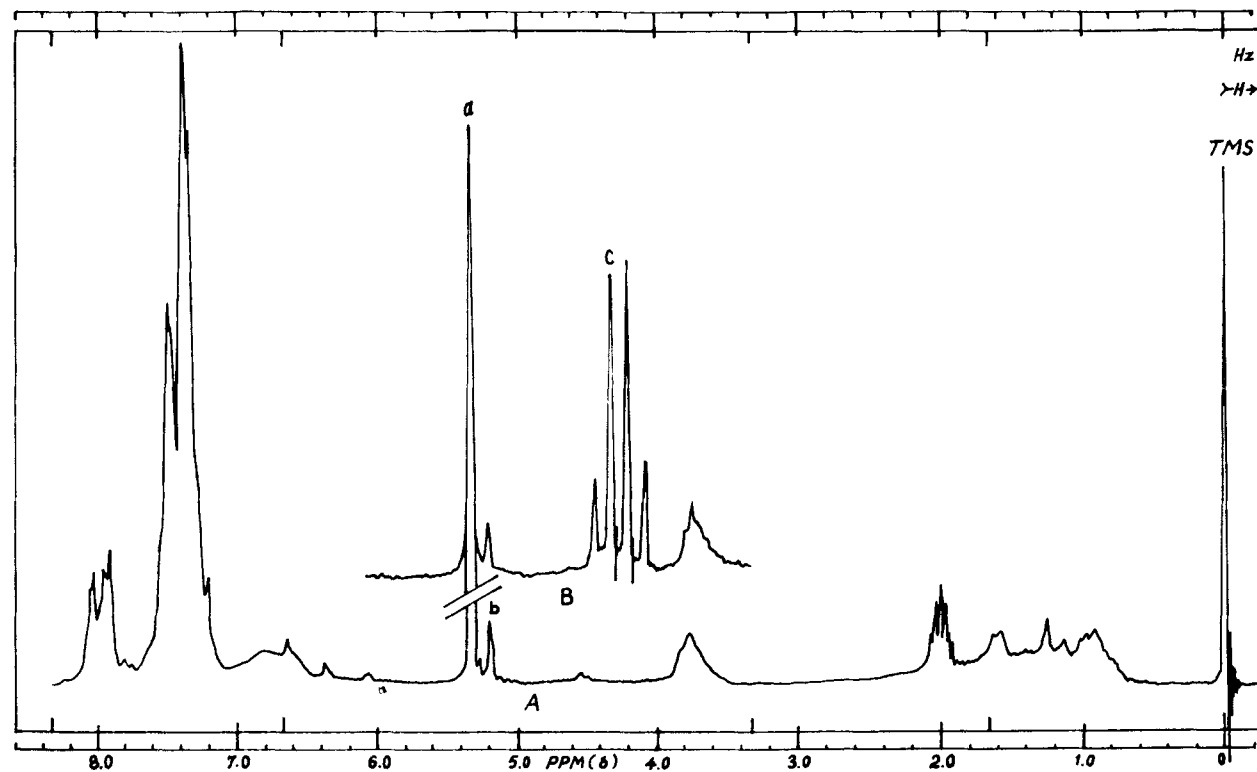


Fig. 7: A. PMR spectrum of Peru balsam in acetone-D₆.

B. Part of the PMR spectrum of peru balsam and ethyl benzoate in acetone.

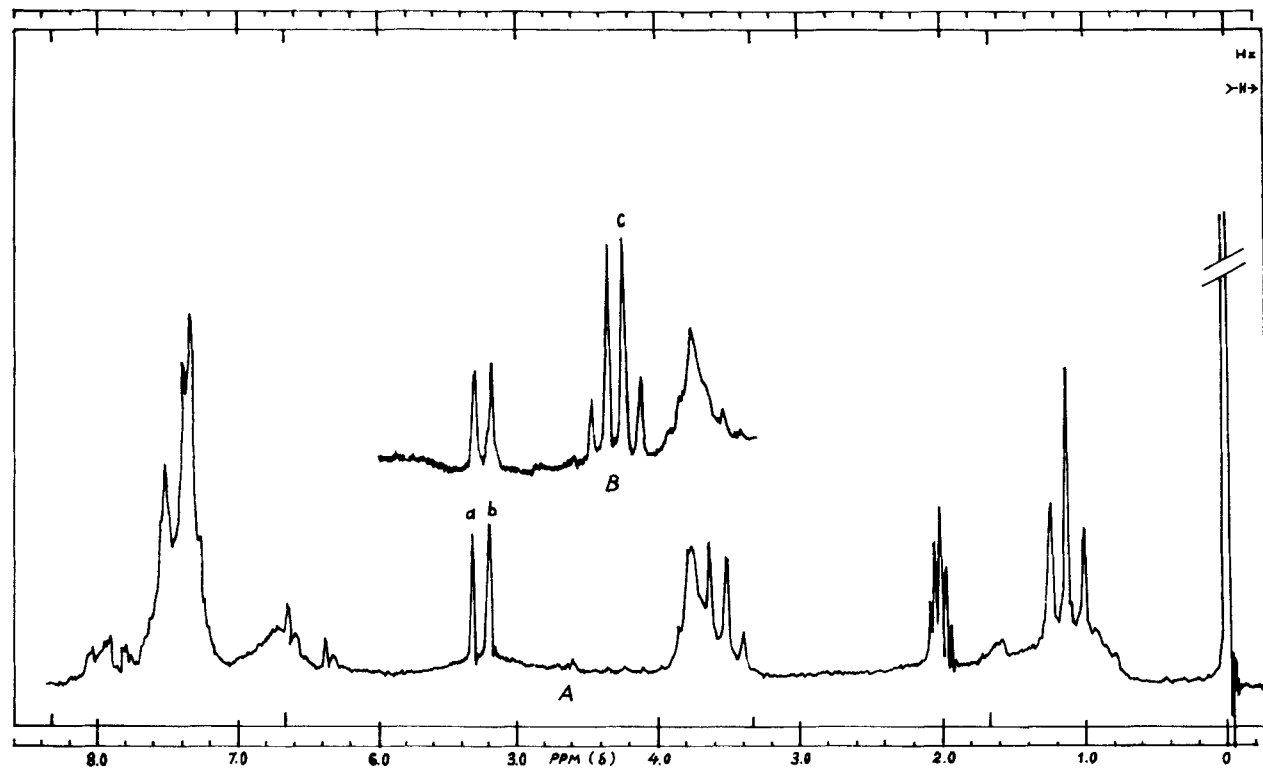


Fig. 8: A. PMR spectrum of Tolu balsam in acetone-D6.

B. Part of the PMR spectrum of Tolu balsm and ethyl benzoate in acetone.

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CLINDAMYCIN HYDROCHLORIDE

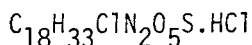
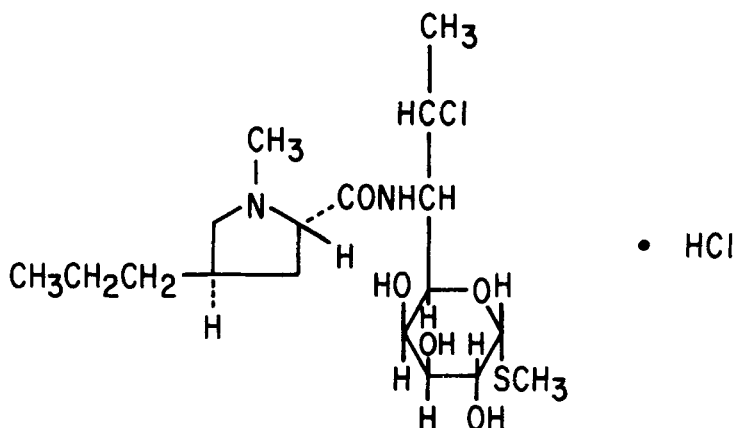
Leo W. Brown and William F. Beyer

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1. Description

1.1 Name, Formula, Molecular Weight

Clindamycin hydrochloride is methyl 7(S)-chloro-6,7,8-trideoxy-6-trans-(1-methyl-4-propyl-L-2-pyrrolidinecarboxamido)-1-thio-L-threo- α -D-galacto-octopyranoside monohydrochloride¹, also 7 Cl-7-deoxylincomycin.



Mol Wt. 461.44

1.2 Appearance, Color, Taste, and Odor

Clindamycin hydrochloride monohydrate is a white or practically white, crystalline powder. It is odorless or has a faint mercaptan-like odor and has a bitter taste.

2. Physical Properties

2.1 Approximate Solubility²

<u>Solvent</u>	<u>Solubility, mg/ml</u>
Water	<1000, >500
Pyridine	<200, >100
Methanol	<200, >100
Ethyl Acetate	<1

2.1 Approximate Solubility (con't)

<u>Solvent</u>	<u>Solubility, mg/ml</u>
Acetone	<1
Chloroform	<1
Dimethylformamide	<500,>250
Benzene	<1
Cyclohexane	<1
Ethanol	<10,>5

2.2 Melting Range³

Clindamycin Hydrochloride 141-143°C

Epiclindamycin Hydrochloride 164-166°C (7-position)

2.3 Specific Rotation³

Clindamycin Hydrochloride +144°(water)

Epiclindamycin Hydrochloride +122°(water)

2.4 pKa

The pKa of clindamycin hydrochloride has been reported as 7.6⁴, the same as lincomycin⁵.

2.5 Crystal Properties⁶

Crystals of clindamycin hydrochloride monohydrate were determined to be monoclinic with cell parameters:

$a = 9.47\text{\AA}$, $b = 9.91\text{\AA}$, $c = 13.50\text{\AA}$, $\beta = 104.5^\circ$. The crystal structure is stabilized by complex hydrogen bonding involving the chloride ion and the water of hydration.

2.6 Infrared Spectrum

A typical mineral oil mull spectrum of clindamycin hydrochloride monohydrate is shown in Figure 1. Anhydrous clindamycin hydrochloride is hygroscopic, consequently, different crystal forms have been observed due to the degree of hydration⁷. Solvation with ethanol or acetone is also possible, producing variation in the infrared spectrum.

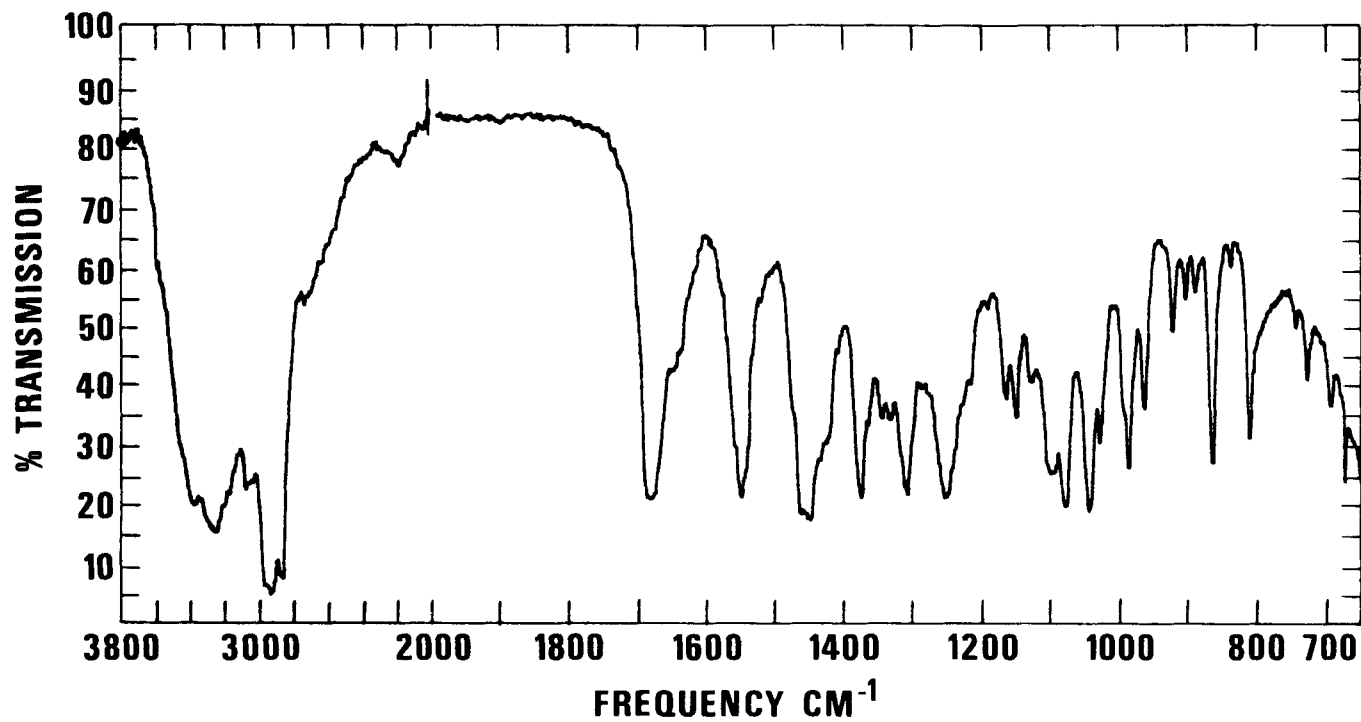


Figure 1. Infrared spectrum of clindamycin hydrochloride monohydrate.

2.7 Nuclear Magnetic Resonance Spectrum

Slomp and MacKellar⁸ used nmr to study the structure of lincomycin, its degradation products, and some analogs including clindamycin (7-chloro-7-deoxy-lincomycin). They found the nmr spectrum of lincomycin to be complex, containing many superimposed multiplets which were difficult to factor. Consequently, the compound was hydrolyzed at the amide linkage into an amino acid (L-trans-4-n-propylhygric acid) and an amino thio sugar. Values obtained for the sugar moiety of clindamycin are shown in Table I.

The nmr spectrum of clindamycin is quite similar to that of lincomycin with the exception of a downfield shift of the doublet attributable to the hydrogens of the terminal methyl(C-8) due to the chlorine atom at the 7-position of clindamycin instead of the hydroxyl at this position in lincomycin.³

TABLE I

Chemical shifts and coupling constants obtained on the sugar moiety of clindamycin in D₂O with sodium 2,2-dimethyl-2-silapentane-5-sulfonate as reference.

<u>Position</u>	<u>Chemical Shift</u>	<u>Coupling Constant</u>	
	<u>Frequency, cps</u>	<u>J_{xy}</u>	<u>Frequency, cps</u>
C-1	325	1,2	5.5
C-2	249	2,3	10.5
C-3	219	3,4	3.5
C-4	235	4,5	1
C-5	260	5,6	10.5
C-6	264	6,7	1.5
C-7	272	7,8	7.0
C-8	87		

Carbon - 13 nmr spectral analysis and spin - lattice relaxation times for clindamycin hydrochloride were reported by Mizzsak et al.⁹

2.8 Mass Spectrum

Kagan and Grostic¹⁰ used mass spectrometry as a technique for structural determination of lincomycin, its degradation products and analogs including clindamycin. They found clindamycin to have essentially the same fragmentation pattern as lincomycin. Some characteristic m/e values and their percent relative intensity (in brackets) were reported as follows:

[M] ⁺	(a)	(b)	(c)	(d)
424(0.3)	377(0.4)	275(3.4)	126(100.0)	82(3.5)
426(0.2)	379(0.1)	277(1.3)		

Fragments containing the chlorine atom produce two values due to the two isotopes of the chlorine atom ($^{35}_{17}\text{Cl}$, $^{37}_{17}\text{Cl}$). Figure 2 shows the structures of the characteristic fragment ions.

3.0 Synthesis and Proof of Structure

Clindamycin(7(S)-chloro-7-deoxylincomycin) can be prepared by treating lincomycin hydrochloride with thionyl chloride or with triphenylphosphine (along with chlorine or carbontetrachloride).³ The reactions place a chlorine atom at the 7-position of lincomycin replacing the hydroxyl at that position with an inversion of configuration. Configuration of the hydroxyl at C-7 in lincomycin was previously established as 7(R). Since the methods used for synthesis of clindamycin are known to cause inversion, the expected configuration of the chlorine at the 7-position was 7(S). Oxidation of clindamycin produced an acidic fragment identified as L-chloropropionic acid giving unequivocal evidence as to the configuration at C-7.

4.0 Drug Metabolites

Microbial transformations of clindamycin were studied by Coats and Argoudelis.¹¹ They showed that clindamycin added to whole-cell cultures of Streptomyces coelicolor resulted in production of the inactive (against Sarcina lutea) metabolite clindamycin 3-phosphate. Clindamycin sulfoxide was the major transformation when clindamycin was added to fermentations of Streptomyces armentosus.¹¹ N-demethylation was observed in fermentations of Streptomyces punipalus.

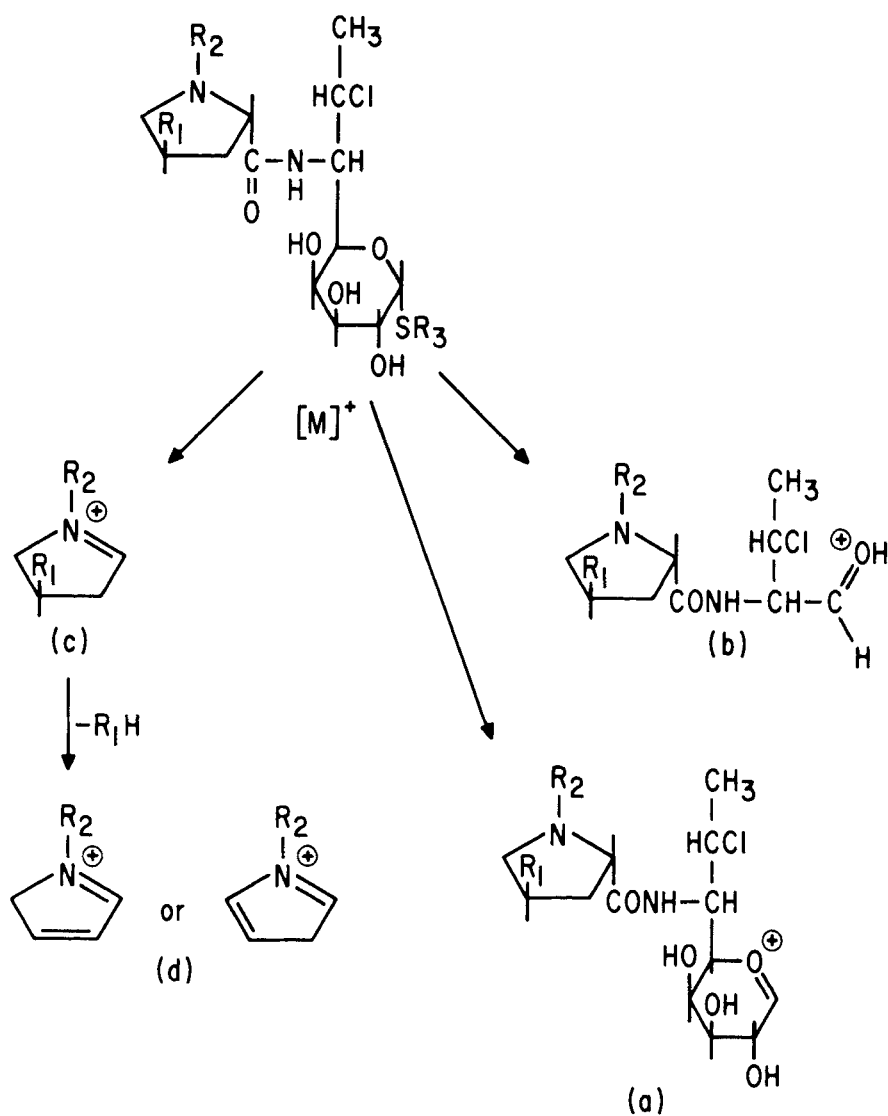


Figure 2. Fragmentation pattern of clindamycin.

R_1 = n-propyl, R_2 , R_3 = methyl

4.0 Drug Metabolites (con't)

In human subjects, a metabolite believed to be the N-demethylation compound was detected by Brodasky et al¹³ using thin layer chromatography. Using radioactive clindamycin given orally to a dog, Sun¹⁴ determined that the following radioactive materials were excreted in the dog urine: 36% intact drug, 28% as the sulfoxide, and 28% as the glucuronide conjugate of the intact drug. In an experiment using the rat, he found that the following were excreted in the urine: 53% as intact drug, 31% as the sulfoxide, and 15% as the N-demethyl metabolite.

5.0 Pharmacokinetics and Toxicity

Absorption, excretion and half-life of clindamycin in normal adult males was studied by Wagner et al.¹⁵ Clindamycin was absorbed extremely rapidly in man following oral administration. The half-life of clindamycin, estimated from serum activities, averaged 2.38 hours. Approximately one-eighth of the dose of clindamycin administered orally was recovered as active drug in the urine in 24 hours.¹⁶ Pharmacokinetic studies in humans were also made by DeHaan et al.¹⁷ They found no evidence of drug accumulation or that it stimulates its own metabolism during repeated dosing.

Common untoward effects of clindamycin given orally in man were loose stools, cramps, excessive flatus, and nausea. Oral toxicity of clindamycin in laboratory animals was studied by Gray et al.¹⁸ They found the maximum daily tolerated dose in the dog and rat for as long as one year was greater than 300 mg/kg but less than 600 mg/kg. At 600 mg/kg in short term studies, the effects of focal irritation in the mucosa of the stomach and gallbladder of the dog were in evidence.

6.0 Antibacterial Activity

Garrison et al¹⁹ have compared in-vitro antibacterial activities of clindamycin, lincomycin, and erythromycin against a number of strains of several genera of clinical isolates of bacteria. Oppenheimer and Turck²⁰ carried out both laboratory and clinical evaluation of clindamycin showing it to be active against Staphylococcus aureus and Diplococcus pneumoniae but of minimal activity against Enterobacteriaceae. Magerlein²¹ has reviewed the antibacterial activity of clindamycin and its microbial modifications.

Structure-activity relationships of lincomycin, clindamycin, and related antibiotics have been reviewed by Pyke.²²

7.0 Methods of Analysis

7.1 Microbiological

Hanka et al²³ developed a microbiological assay for lincomycin which is also applicable to clindamycin. It is a disk-plate agar diffusion method using Sarcina lutea as the assay organism. The procedure is sensitive to approximately 0.25 mcg/ml in blood-level determinations. Sarcina lutea is grown for 18 hours on the surface of agar in Roux bottles, harvested, and suspended in a broth at a concentration of about 3×10^{10} cells per ml. Penassay Seed Agar is inoculated with the cell suspension at 0.5 ml of the culture per liter of medium. Eight ml of the seeded medium is poured into a plastic petri dish and allowed to solidify. A sample of a buffered solution of clindamycin, approximately 5 mcg/ml, is applied on an assay disk and the plate incubated for 16-18 hours at 30°C. The zone of inhibition is then measured and compared with a reference.

7.2 Paper Chromatographic

Paper chromatography has been widely used in identification of antibiotics from fermentation beers. Mason et al²⁴ used six solvent systems (Table II) to characterize lincomycin, a precursor of clindamycin. Zones were located and quantitated by bioautography on Sarcina lutea.

TABLE II

Solvent systems used for identification of antibiotics by paper chromatography.

	<u>Developing Solvent</u>	<u>Developing Time, hr.</u>
I	1-butanol-water(84:16)	16
II	1-butanol-water(84:16) plus 0.25% p-toluenesulfonic acid	16
III	1-butanol-acetic acid-water (2:1:1)	16
IV	1-butanol-water(84:16) plus 2% piperidine	16

7.2 Paper Chromatographic (con't)

	<u>Developing Solvent</u>	<u>Developing Time, hr.</u>
V	1-butanol-water(4:96)	5
VI	1-butanol-water(4:96) plus 0.25% p-toluenesulfonic acid	5

7.3 Gas Chromatographic

Stability of clindamycin in aqueous solution was determined by Oesterling²⁵ using gas chromatography. Brown²⁶ developed a gas chromatographic assay for clindamycin in hard filled capsules. For satisfactory chromatography, derivatization (trimethylsilyl, acetyl, trifluoroacetyl) was usually employed to neutralize the polar groups of the clindamycin molecule. The trifluoroacetic acid derivative was prepared by adding 0.5 ml of trifluoroacetic anhydride to approximately 5 mg of clindamycin hydrochloride in 2.0 ml of chloroform containing hexacosane as internal standard. The solution was heated at 45°C for 30 min. and chromatographed on a 61 cm column packed with 3% OV-17 on 60-80 mesh Gas Chrom Q. Figure 3 shows a chromatogram obtained using the following conditions: column temperature 170°C, FID detector temperature 200°C, helium carrier gas at a flow of 60 ml/min., and 1 microliter sample volume injected directly into the glass column.

7.4 Liquid Chromatographic

Brown²⁷ chromatographed clindamycin hydrochloride by HPLC using ion-pair chromatography. Figure 4 shows a chromatogram obtained using the following conditions:

Column: 30 cm x 4 mm ID prepacked with C₁₈
μBondapak

Mobile Phase: 1 g. sodium dioctyl sulfosuccinate
1.0 ml formic acid
125 ml water
q.s. 500 ml with anhydrous methanol

Detection: refractive index detector (9.6×10^{-5}
RI units full scale)

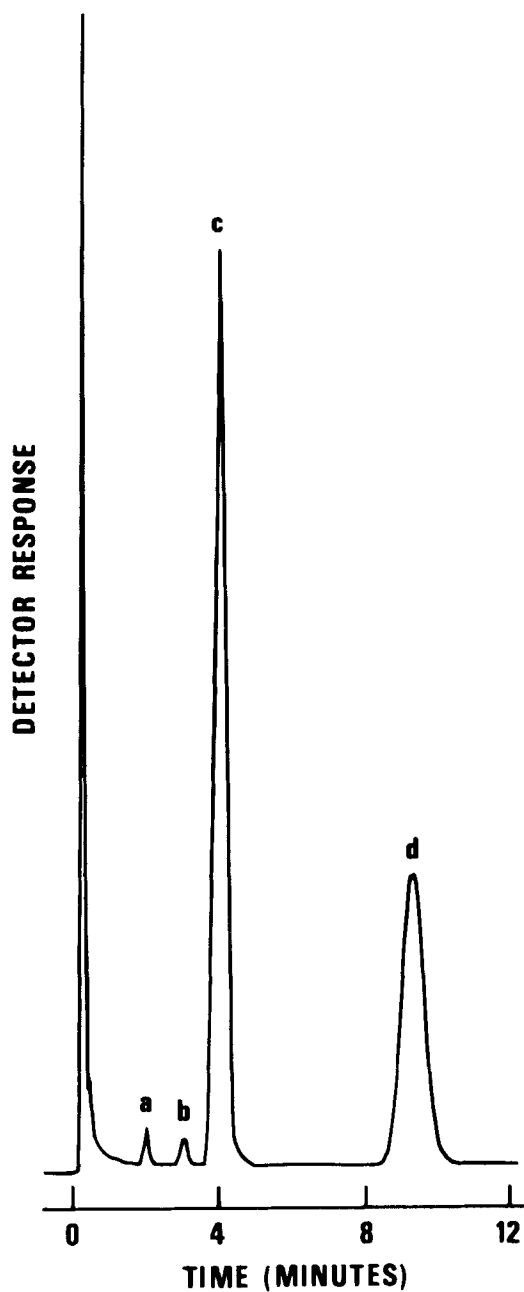


Figure 3. Gas chromatogram of: (a) trifluoroacetylated epilincosmycin, (b) trifluoroacetylated clindamycin B, (c) trifluoroacetylated clindamycin, and (d) hexacosone as internal standard.

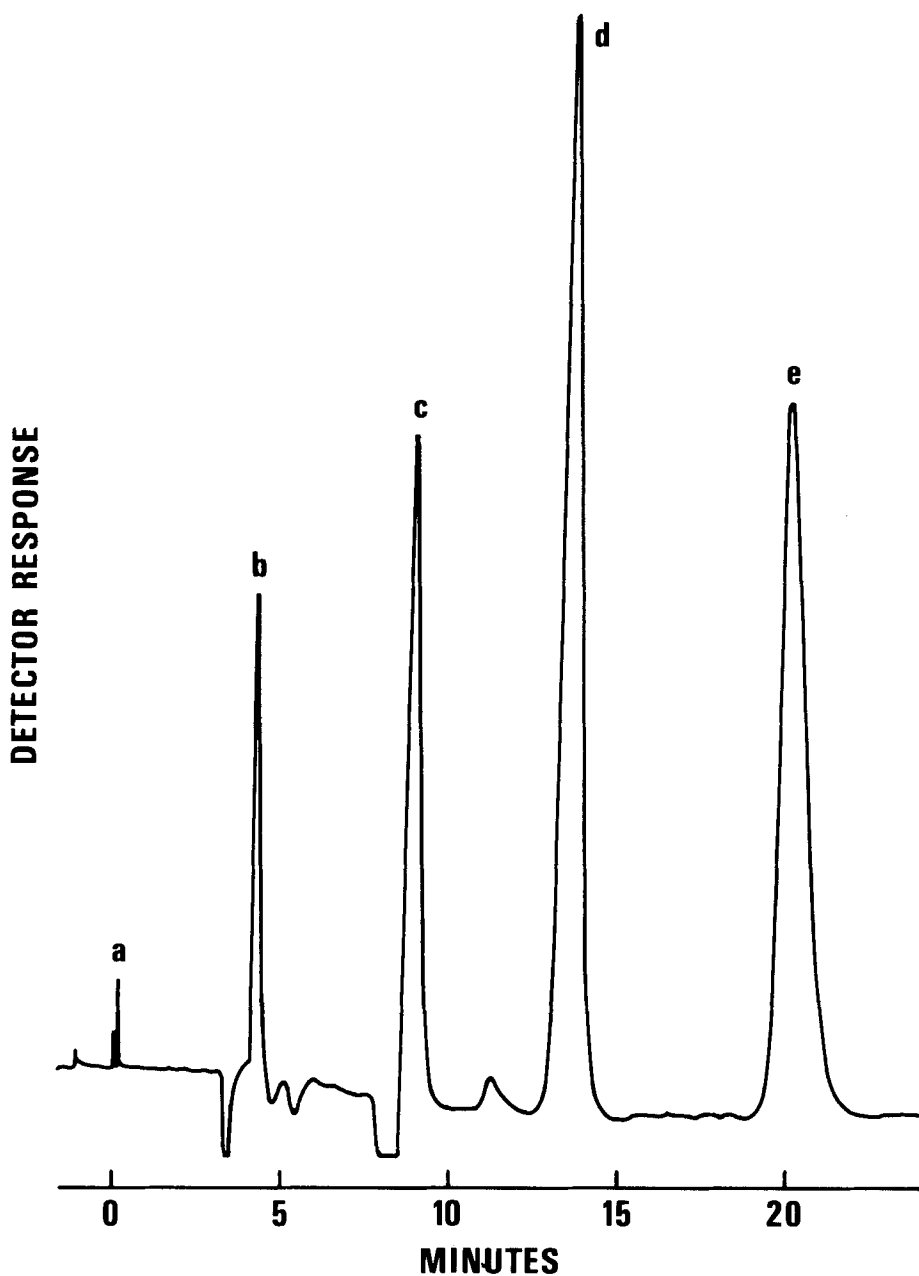


Figure 4. HPLC chromatogram of: (a) injection, (b) solvent, (c) lincomycin, (d) clindamycin, (e) testosterone propionate as internal standard.

7.4 Liquid Chromatographic (con't)

Column Pressure: 1000 psi

An improved ion-pairing HPLC method has been developed by Landis²⁸ which separates clindamycin, clindamycin B, and 7-epiclindamycin. (In Brown's method²⁷, epi-clindamycin and clindamycin elute as a single peak.) RI detection was utilized with the mobile phase consisting of a 60/40 ratio methanol/water, 2 ml glacial acetic acid per liter (0.035 M), and 0.005 M D,L-10-sodium camphor sulfonate, adjusted to pH 6.0. A 30 cm x 3.9 mm Water's μ Bondapak[®] C₁₈ column was utilized at a flow rate of 1.0 ml/min. (approximately 900 psig). Data are given for the assay of capsules, syrups, and clindamycin hydrochloride bulk drug. The RSD for the assay is approximately 1%. A chromatogram of a synthetic mixture containing lincomycin, lincomycin B, clindamycin B, and 7-epiclindamycin, and clindamycin is given in Figure 5. The author stated that if additional sensitivity is required UV 214 nm detection can be employed, utilizing a mobile phase comprised of a 60/40 ratio methanol/water, 0.01 M phosphate buffer, and 0.005 M sodium pentane sulfonate.

7.5 Titrimetric

Clindamycin hydrochloride has been titrated with 0.5 N potassium hydroxide using a potentiometric titrator.²⁹ The endpoint is the point at which $\Delta E/\Delta V$ is greatest.

7.6 Radioimmunoassay

A radioimmunoassay was developed by Gilbertson and Stryd³⁰ which is as sensitive as the microbiological assay (approximately 0.1 mcg/ml). Tritiated clindamycin was prepared for the assay by reacting N-demethyl clindamycin with formaldehyde and tritium sodium borohydride. Clindamycin-2-hemisuccinate was prepared and coupled with bovine serum albumen. Rabbits were injected with the conjugate along with Freund's Complete Adjuvant at monthly intervals to product antiserum. Serum samples to be assayed for clindamycin were extracted with chloroform and the chloroform extract evaporated to dryness. Standards were made in the range of 1 to 250 nanograms of clindamycin hydrochloride per 0.1 ml of distilled water.

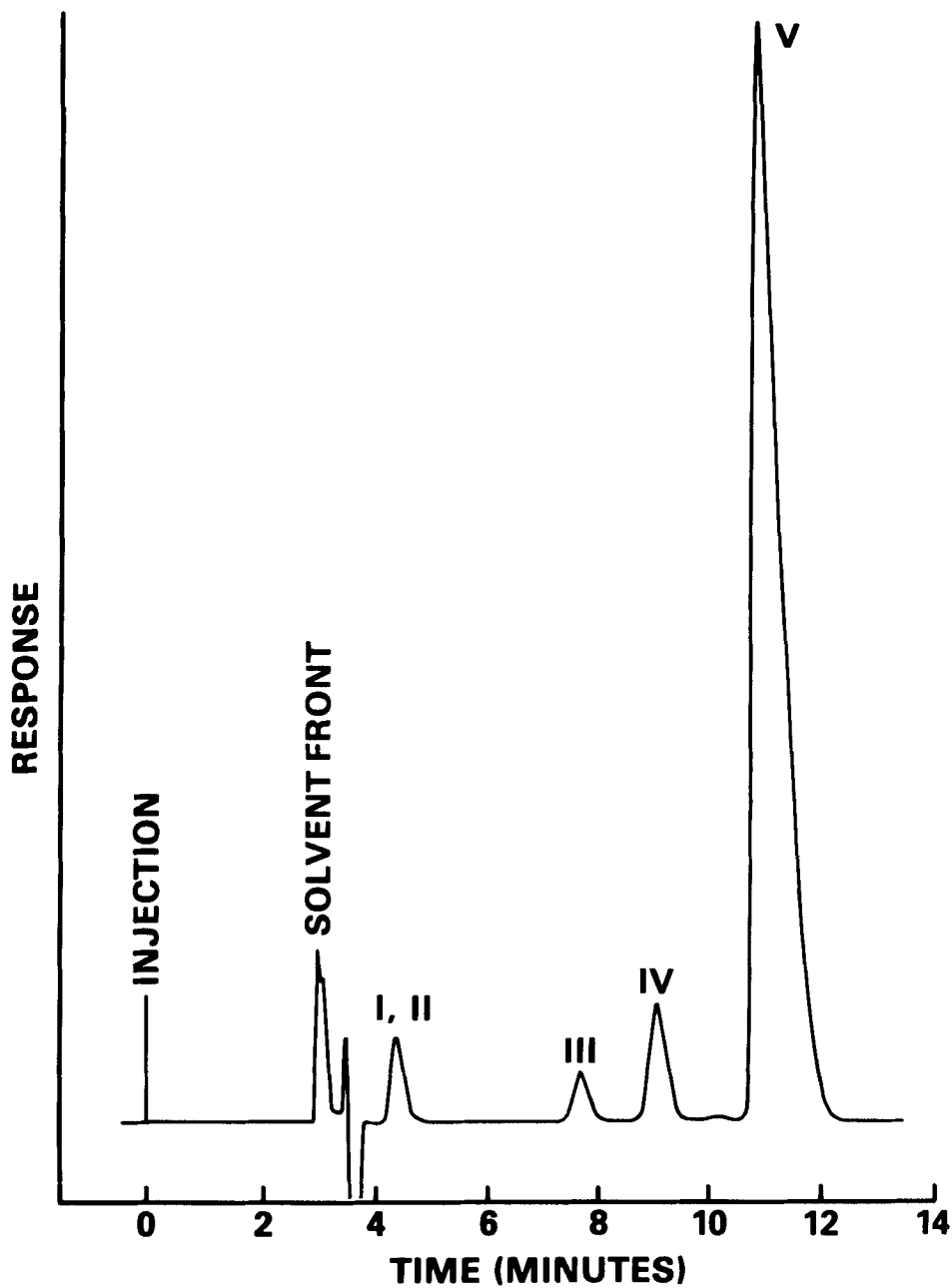


Figure 5. Chromatogram of a synthetic mixture of lincomycin (I), lincomycin B (II), clindamycin B (III), 7-epiclindamycin (IV), and clindamycin (V).

7.6 Radioimmunoassay (con't)

The assay procedure involves the double antibody method in which both unlabeled and tritiated clindamycin compete for binding sites on the antibodies of the antiserum produced from the rabbits. A second antibody is added to form an insoluble primary-secondary antibody complex. The precipitate is washed and scintillation cocktail added. After dispersion of the precipitate in the cocktail, the mixture is counted in a liquid scintillation counter.

7.7 Thin Layer Chromatographic

Silica gel H thin layer plates with developing solvent methyl ethyl ketone-acetone-water(186:52:20) were used by Brodasky et al¹³ to quantitate the N-demethyl clindamycin metabolite. The zones were detected by bioautography on *Sarcina lutea*. Silica gel GF₂₅₄ thin layer plates have been used with developing solvent of methanol-chloroform (1:3).² In this system, clindamycin has an approximate R_f of 0.70 while lincomycin has an R_f of 0.65. Detection is usually by charring, iodine vapors, or permanganate spray.

The latter consists of 10 g. of potassium carbonate, 8 g. of sodium periodate, and 1 g. of potassium permanganate in 500 ml of water. The mixture is left standing for 16 hours and filtered. Clindamycin gives a yellow spot on a purple background using this spray.

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CODEINE PHOSPHATE

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and
Mahmoud M. A. Hassan

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1. Description

1.1. Nomenclature

1.1.1 Chemical Names

(a) 7,8-Didehydro-4,5 α -epoxy-3-methoxy-17-methylmorphinan-6 α -ol-phosphate (1 : 1) (salt).

(b) Morphinan-6-ol, 7,8-didehydro-4,5-epoxy-3-methoxy-17methyl-, (5 α , 6 α) phosphate (1 : 1) (salt).

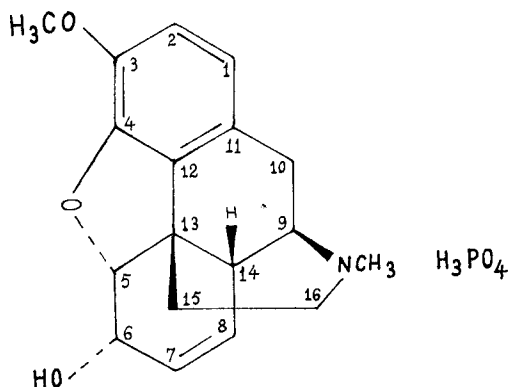
1.1.2 Generic Names

Codeine phosphate ; Morphine-3-methyl ether phosphate; Methymorphine phosphate; Morphine monomethyl ether phosphate.

1.2. Formulae

1.2.1 Emprical: $C_{18}H_{24}NO_7P$. (Anhydrous)
 $C_{18}H_{24}NO_7P \cdot \frac{1}{2}H_2O$ (Hemihydrate)
 $C_{18}H_{24}NO_7P \cdot \frac{3}{2}H_2O$ (Sesquihydrate)

1.2.2 Structural



More than twenty structures were proposed for morphine and other related phenanthrene alkaloids. The currently accepted structure is that proposed in 1925 by Gulland and Robinson (1). The proposed structure was confirmed by the total synthesis of morphine in 1956 which was achieved by Gates and Tschudi (2).

1.2.3 CAS no.

[52-28-8] codeine phosphate trihemihydrate
 $(C_{18}H_{21}NO_3 \cdot H_3PO_4 \cdot 3/2 H_2O)$

1.2.4 Wiswesser Line Notation

TB6 566 B6/CO 4ABBC R
BX H \bar{O} PN DU GHT & & TTJ
FQ J01 P & QH & H3-P- $\bar{O}4$

1.2.5 Stereochemistry

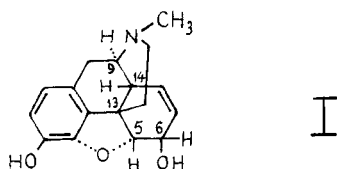
Codeine possesses five different asymmetric centres (at C_5 , C_6 , C_9 , C_{13} and C_{14}), but since the bridged ring system imposes some rigidity upon the system, the theoretical number of optical isomers is limited to sixteen. The deduction of relative configurations at the various centres by chemical methods has been well summarized by Ginsburg (3).

The absolute stereochemistry has been deduced from a combination of X-ray crystallography and chemical degradation and correlation I.

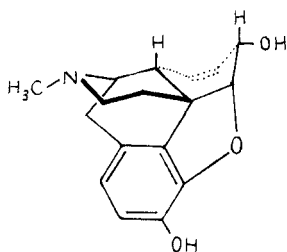
From the X-ray data of morphine hydroiodide dihydrate (4) and of codeine hydrobromide dihydrate (5,6) it was concluded that the molecules are approximately T-shaped, with atoms of rings A and B and the oxide ring lying near one plane, and the atoms of rings C(cycloalkene ring) and D(piperidine ring) lying close to a second plane at right angles to the first. The B/C ring junction was shown to be cis, and piperidine ring, D,

to be an almost regular chair-shape, with the methyl group attached to nitrogen by an equatorial bond. The cycloalkene ring C is almost boat-shaped, and the C₅-oxygen and C₆-hydroxyl group are cis to each other (7).

The absolute geometry of the entire molecule II, follows from its relative stereochemistry and has been confirmed by optical rotatory dispersion studies (8).



I



II

1.3 Molecular Weight:-

Anhydrous	397.37
Hemihydrate	406.36
Sesquihydrate	424.37

1.4 Elemental Composition:

Anhydrous

C, 54.41% ; H, 6.05% ; O, 28.18% ; P, 7.80%
N, 3.56%.

Hemihydrate

C, 53.20% ; H, 6.16% ; N, 3.45% ; O, 29.56% ,
P, 7.63%

Sesquihydrate

C, 50.94% ; H, 6.37% ; N, 3.30% ; O, 32.08% ; P, 7.31%

1.5 Appearance, Color, Odor and Taste:-

Codeine phosphate occurs in two forms, one containing $\frac{1}{2}$ molecule of water of crystallization and the other containing $1\frac{1}{2}$ molecule of water of crystallization. It is odorless and has a bitter taste.

Hemihydrate

Fine, white, needle shaped crystals or white crystalline powder.

Sesquihydrate

Very efflorescent, small crystals or crystalline powder.

2. Physical Properties

2.1.1 X-ray Diffraction

The stereochemical configuration of the codeine molecule was determined by Lindsey and Barnes (5) by a two dimensional study of codeine hydrobromide dihydrate. A three dimensional study of the salt has been also carried out by Kartha et al (6). Interatomic distances and bond angles are listed in Tables 1 and 2 respectively. The codeine molecule in its absolute configuration is represented in Fig.1.

Table 1. Interatomic Distances (\AA^0)

<u>Intramolecular</u>					
$\text{CH}_3'-\text{O}_1$	1.443	(1.58)	$\text{C}_{14}-\text{C}_8$	1.497	(1.48)
O_1-C_3	1.399	(1.49)	C_8-C_7	1.324	(1.31)
C_3-C_2	1.372	(1.45)	C_7-C_6	1.531	(1.49)
C_2-C_1	1.401	(1.34)	C_6-C_5	1.516	(1.59)
C_1-C_{11}	1.387	(1.30)	C_6-OH	1.427	(1.46)
$\text{C}_{11}-\text{C}_{12}$	1.399	(1.40)	$\text{C}_{14}-\text{C}_9$	1.561	(1.59)
$\text{C}_{12}-\text{C}_4$	1.369	(1.32)	C_9-C_{10}	1.539	(1.63)
C_4-C_3	1.388	(1.37)	$\text{C}_{10}-\text{C}_{11}$	1.496	(1.52)

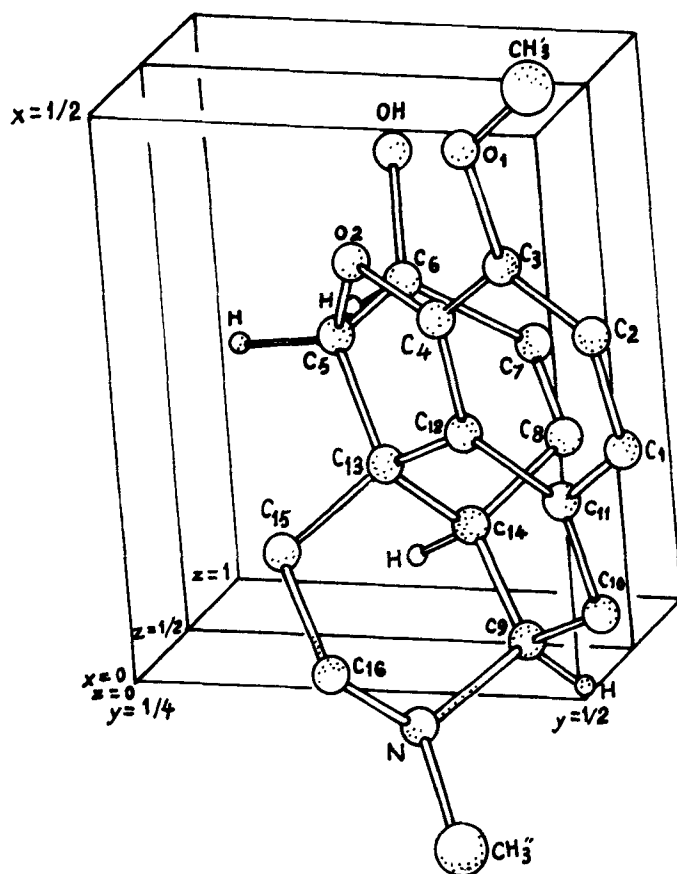


Fig. 1. Model showing the absolute configuration of the codeine molecule (only those H's on C₅, C₆, C₉, C₁₄ are shown).

C ₄ -O ₂	1.370	(1.45)	C ₁₃ -C ₁₅	1.535	(1.47)
O ₂ -C ₅	1.472	(1.40)	C ₁₅ -C ₁₆	1.530	(1.46)
C ₅ -C ₁₃	1.532	(1.62)	C ₁₆ -N	1.468	(1.52)
C ₁₃ -C ₁₂	1.504	(1.54)	N-C ₉	1.521	(1.50)
C ₁₃ -C ₁₄	1.564	(1.58)	N-CH ₃ "	1.506	(1.56)

Intermolecular

C ₁ -C ₇	3.716	C ₉ -OH	3.577	C ₁₆ -O ₂	3.427
C ₂ -C ₇	3.728	C ₁₀ -H ₂ O"	3.680	CH ₃ -H ₂ O"	3.414
C ₃ -H ₂ O'	3.561	C ₁₁ -C ₇	3.722	CH ₃ "-OH	3.463
C ₄ -H ₂ O'	3.528	C ₁₂ -C ₇	3.638	N-C ₆	3.817
C ₅ -Br	3.728	C ₁₃ -H ₂ O'	3.834	O ₁ -C ₁₅	3.708
C ₆ -N	3.817	C ₁₄ -H ₂ O'	3.543	O ₂ -H ₂ O'	2.922
C ₇ -C ₁₂	3.638	C ₁₅ -OH	3.644	OH-CH ₃ "	3.463
C ₈ -H ₂ O"	3.393				

Table 2. Bond angles (°)

CH ₃ -O ₁ -C ₃	117.2	(112)	C ₁₂ -C ₁₃ -C ₁₄	105.5	(105)
O ₁ -C ₃ -C ₄	114.5	(111)	C ₁₅ -C ₁₃ -C ₁₄	108.4	(107)
O ₁ -C ₃ -C ₂	127.3	(129)	C ₅ -C ₁₃ -C ₁₄	116.9	(108)
C ₄ -C ₃ -C ₂	118.2	(119)	C ₁₂ -C ₁₃ -C ₁₅	112.2	(120)
C ₃ -C ₂ -C ₁	120.2	(115)	C ₁₃ -C ₁₄ -C ₈	108.1	(112)
C ₂ -C ₁ -C ₁₁	122.5	(129)	C ₁₃ -C ₁₄ -C ₉	107.8	(108)
C ₁ -C ₁₁ -C ₁₂	115.2	(113)	C ₈ -C ₁₄ -C ₉	112.5	(112)
C ₁₀ -C ₁₁ -C ₁	125.8	(129)	C ₁₃ -C ₁₅ -C ₁₆	112.6	(112)
C ₁₀ -C ₁₁ -C ₁₂	118.6	(118)	C ₁₅ -C ₁₆ -N	110.7	(105)
C ₁₁ -C ₁₂ -C ₄	122.7	(125)	C ₁₆ -N-C ₉	113.6	(116)
C ₁₃ -C ₁₂ -C ₁₁	127.3	(126)	C ₁₆ -N-CH ₃ "	110.4	(109)
C ₁₃ -C ₁₂ -C ₄	109.4	(109)	C ₉ -N-CH ₃ "	113.1	(108)
C ₁₂ -C ₄ -C ₃	120.8	(118)	N-C ₉ -C ₁₄	105.2	(109)
C ₁₃ -C ₁₂ -O ₂	111.9	(115)	N-C ₉ -C ₁₀	113.0	(111)

$C_3-C_4-O_2$	127.0	(126)	$C_{14}-C_9-C_{10}$	114.0	(109)
$C_4-O_2-C_5$	107.7	(102)	$C_9-C_{10}-C_{11}$	115.4	(119)
$O_2-C_5-C_{13}$	105.1	(108)	C_5-C_6-OH	112.5	(104)
$C_6-C_5-C_{13}$	112.8	(118)	C_7-C_6-OH	111.2	(107)
$C_6-C_5-O_2$	111.0	(119)	$C_5-C_6-C_7$	113.9	(100)
$C_5-C_{13}-C_{12}$	101.3	(96)	$C_6-C_7-C_8$	119.8	(123)
$C_5-C_{13}-C_{15}$	112.4	(119)	$C_7-C_8-C_{14}$	120.6	(118)

The intramolecular values in parentheses have been recalculated with an IBM 650 computer for the unit cell dimensions at atomic parameters of Kindsey and Barnes (1955).

The interatomic distances and angles suggestive of hydrogen bonding are listed in Table 3.

Table 3. Inateratomic distances and angles involving possible hydrgen bonds (...)

O-H...N	2.772 A	$H_2O' \dots H-Br$	3.262 A
H-O'-H...O-H	2.762	H-Br...H-O''	3.395
H-O'-H...O ₁	2.852	H-Br...H-O''-H	3.397
OH...N-C ₁₆	105.6°	O ₁ ...H ₂ O'...OH	112.9°
OH...N-C ₉	109.3	O ₁ ...H ₂ O'...HBr	102.5
OH...N-CH ₃	104.1	OH... H ₂ O'...HBr	<u>137.8</u>
(C ₁₆ -N-C ₉	113.6)		<u>353.2°</u>
(C ₁₆ -N-CH ₃ ''	110.4)		
(C ₉ -N-CH ₃ ''	<u>113.1)</u>		
Mean	<u>109.3°</u>	$H_2O' \dots HBr \dots H_2O''$	176.6°
		$H_2O' \dots HBr \dots H_2O''$	100.4
		$H_2O'' \dots HBr \dots H_2O''$	<u>82.3</u>
H ₂ O'...OH...N	105.5°		<u>359.3°</u>
H ₂ O'...OH-C ₆	122.3		
C ₆ -OH...N	<u>127.8</u>		

The system of possible hydrogen bond is represented by the broken lines in Fig. 2.

2.1.2. Melting Points

Codeine phosphate melts at:-

245 - 248^o (9) by hot bar method
 225 - 240^o (9) by hot stage method
 220 - 235^o with dec. (10)

2.1.3. Eutectic Temperature

Sal 187^o
 Dic 149^o (9) by hot bar method
 Sal 187^o
 Dic 143^o (9) by hot stage method
 Sal = acetaminosalol Dic = dicyandiamide

2.2. Solubility

Freely soluble in water (1g in 4 ml); very soluble in hot water (1g in 0.5 ml); slightly soluble in alcohol (1g in 450 ml) but more soluble in boiling alcohol (1g in 125 ml) (11, 12)

2.3. Dissociation Constant

The pKa value of codeine phosphate at 20^o is 8.2 (13).

2.4. Optical Rotation

D - 98^o to - 102^o (2% aqueous solution) (13,14).

The optical rotation of codeine phosphate as .013% aqueous solution and as .013 % ethanolic solution have been determined in our laboratory using a Perkin Elmer Polaromatic model 241 MC and found to be:

$$[\alpha]_D^{24} - 110^{\circ}$$

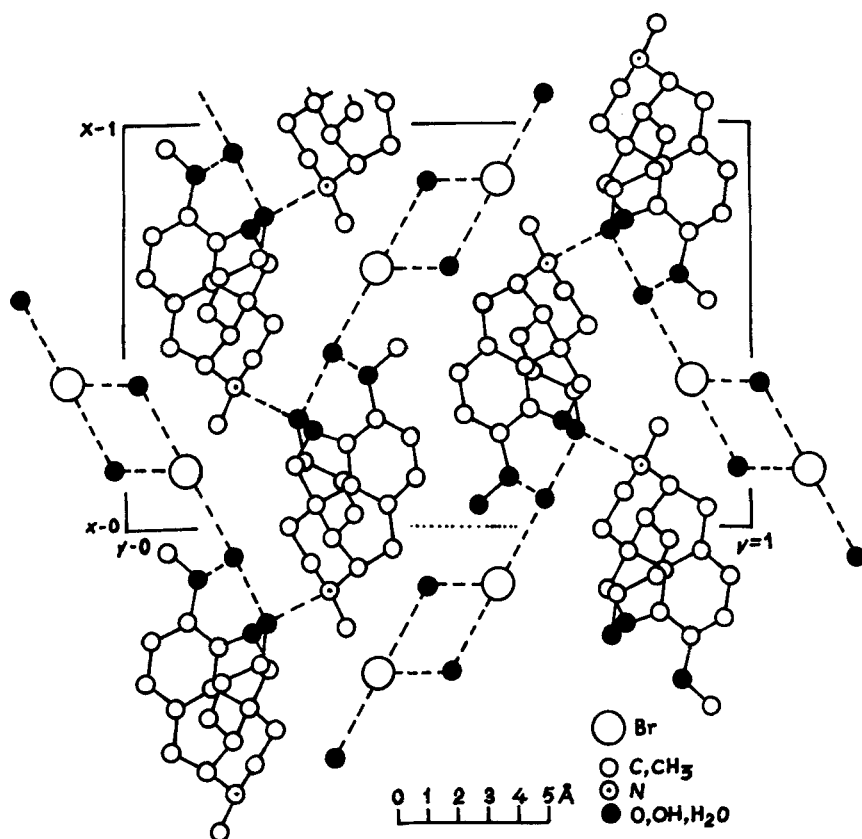


Fig. 2. Projection showing possible hydrogen bonds (broken lines).

2.5. Spectral Properties

2.5.1 Ultraviolet Spectrum

The UV spectrum of codein phosphate in water was scanned from 200 to 400 nm using Varian carry 119 spectrophotometer. It exhibits a characteristic UV spectrum (Fig. 3) with a maximum at 284.8 nm (1570).

Other UV spectral data of codeine phosphate have also been reported:-

λ max 284 nm (1585) in water (15)

λ max 284 nm (E1%, 1 cm about 52.3) in water. (16).

2.5.2 Infra Red Spectrum

The IR spectrum of codeine phosphate as KBr-disc was recorded on a Unicam SP 1025 spectrometer (Fig. 4). The structural assignments have been correlated with the following hand frequencies in Table 4.

Table 4. IR characteristics of codeine phosphate

<u>Frequency Cm^{-1}</u>	<u>Assignment</u>
3500	-OH (broad)
2500	-N ⁺ H
1645	C ₇ =C ₈ (alkene)
1618, 1515	C=C (aromatic)
1280, 1090	C-O-C
790, 760	Two adjacent H (aromatic)

Other characteristic absorption bands are: 2990, 1460, 1335, 960 880 and 845 Cm^{-1} .

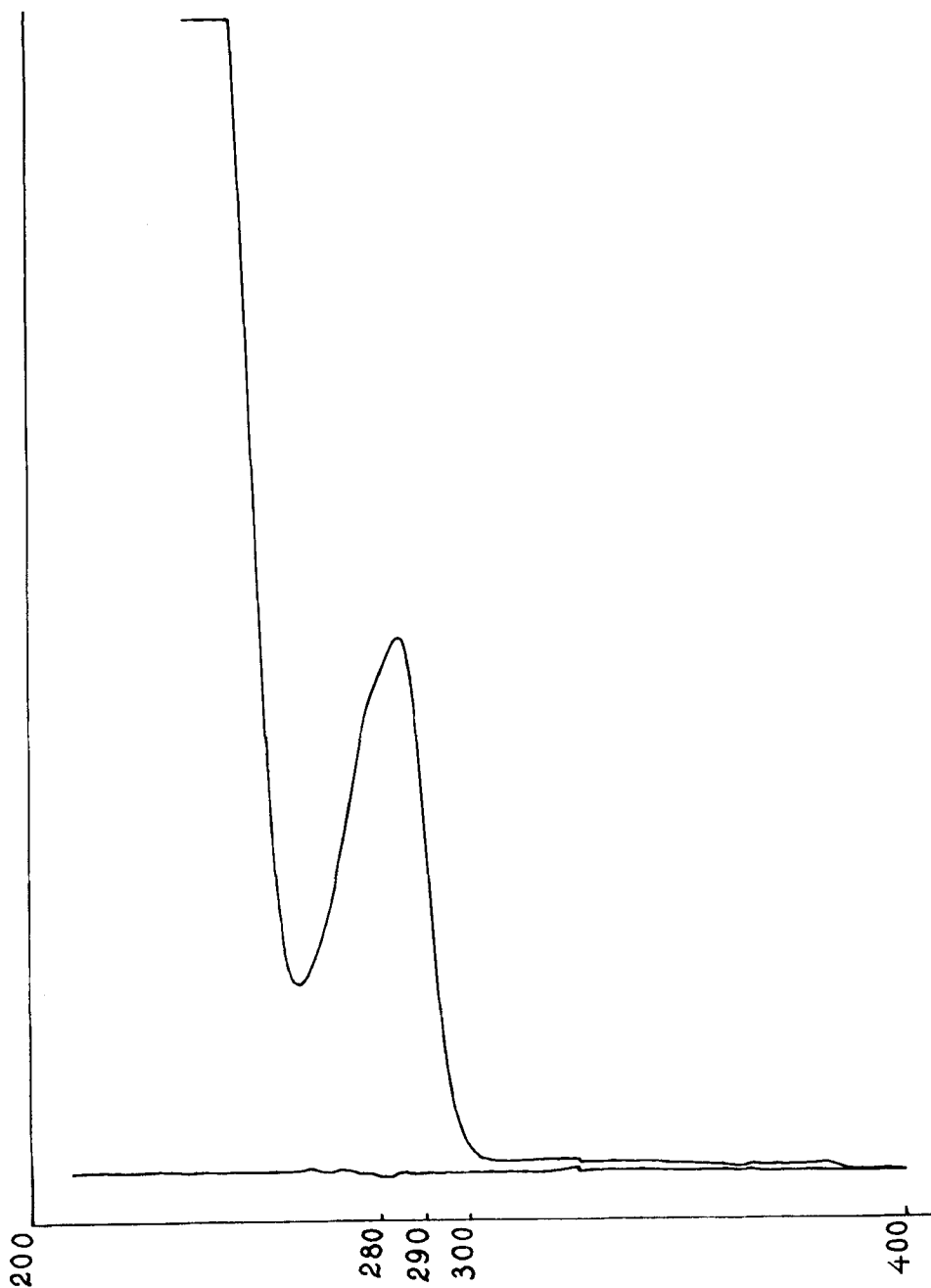


Fig.3. The UV spectrum of codeine phosphate in water.

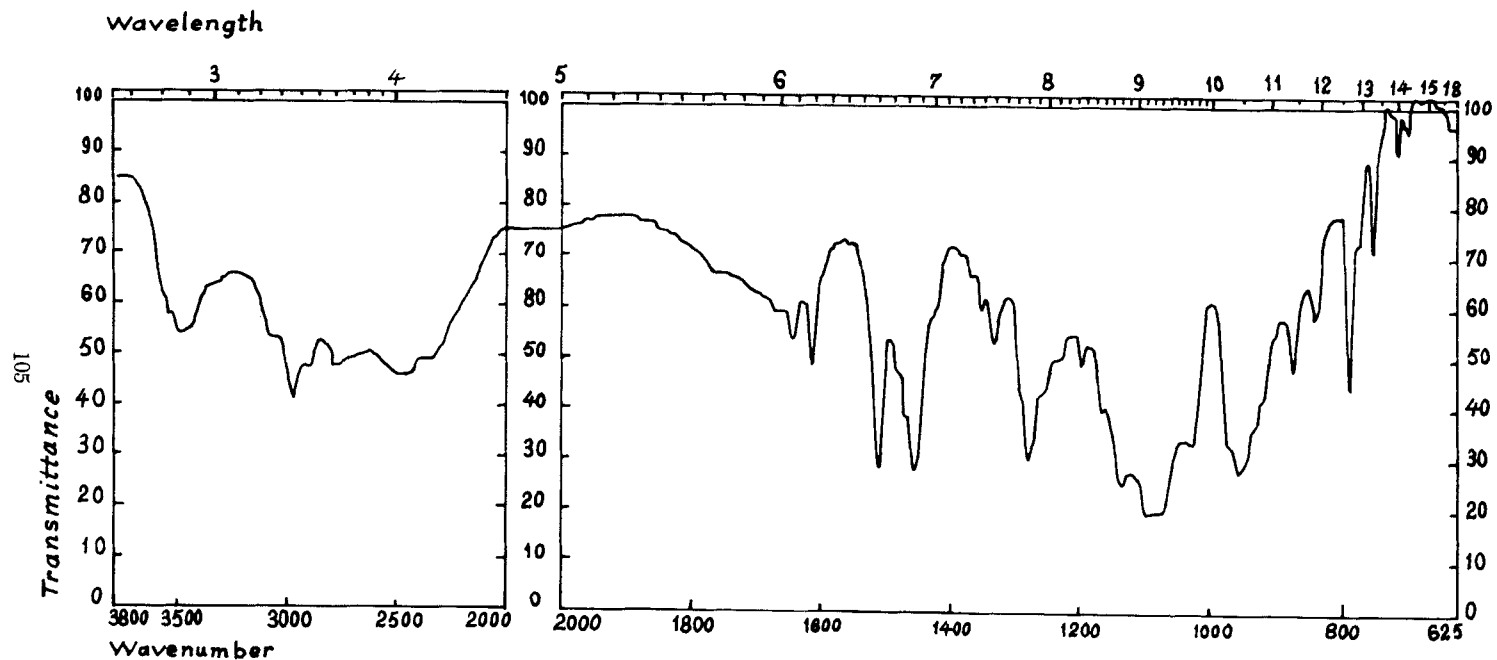


Fig. 4. IR spectrum of codeine phosphate

2.5.3 Nuclear Magnetic Resonance Spectra

2.5.3.1 Proton Spectrum

The PMR spectrum of codeine phosphate in deuterium oxide was recorded on a varian XL 200, 200 MHz NMR spectrometer using tetramethyl silane as a reference standard (Fig. 5). The following structure assignments have been made (Table 5).

Table 5. PMR characteristics of codeine phosphate.

<u>Chemical Shift (δ)</u>		<u>Assignment</u>
6.78	(d)	1H
6.95	(d)	2H
5.78	(d)	7H
5.40	(m)	8H
4.40	(m)	9H
3.87	(s)	3-OCH ₃
3.00	(s)	N -CH ₃

s = singlet, d = doublet, m = multiplet.

2.5.3.2 ¹³C-NMR

¹³C-NMR completely decoupled and off resonance spectra are shown in Fig. 6. (A & B respectively). Both were recorded over 5000 Hz range, in deuterium oxide (conc. 575 mg/2 ml D₂O) on FT-80 A-80 MHz NMR spectrometer. Using 10 mm sample tube and tetramethylsilane as reference standard, at ambient.

The carbon chemical shift are assigned on the basis of the

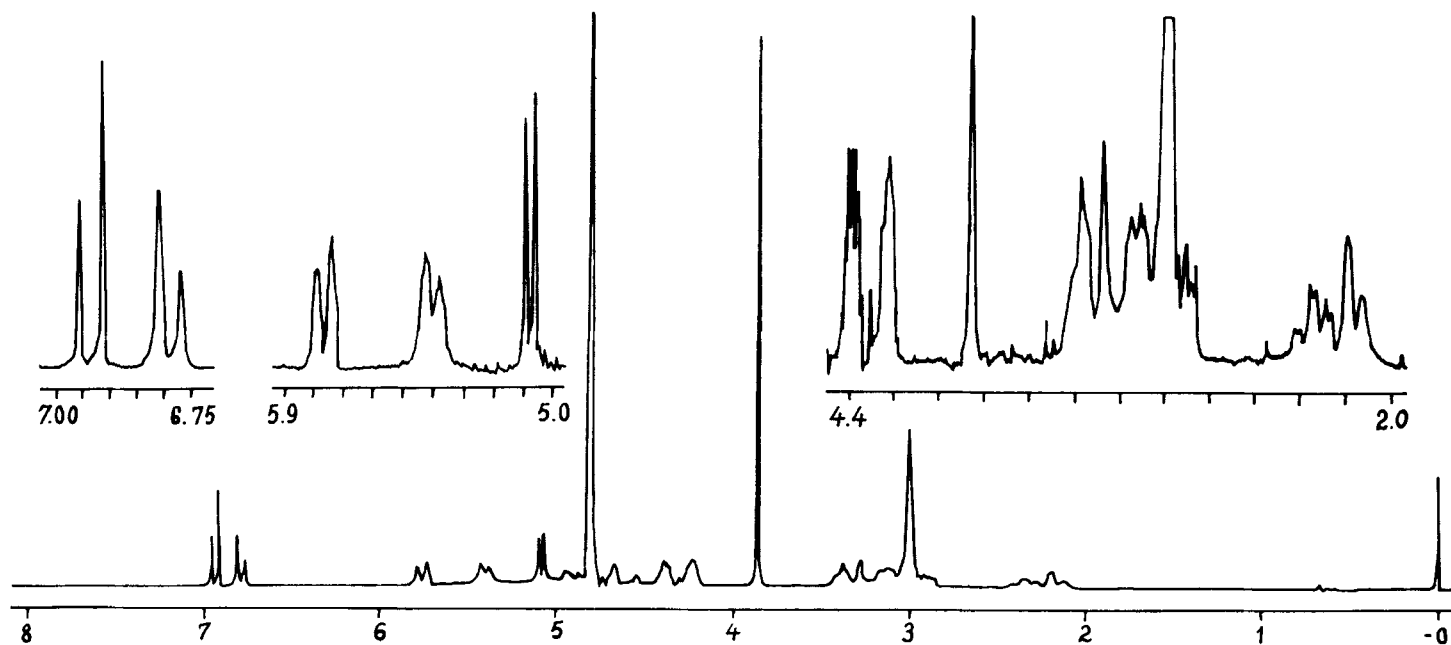


Fig. 5. PMR spectrum of codeine phosphate.

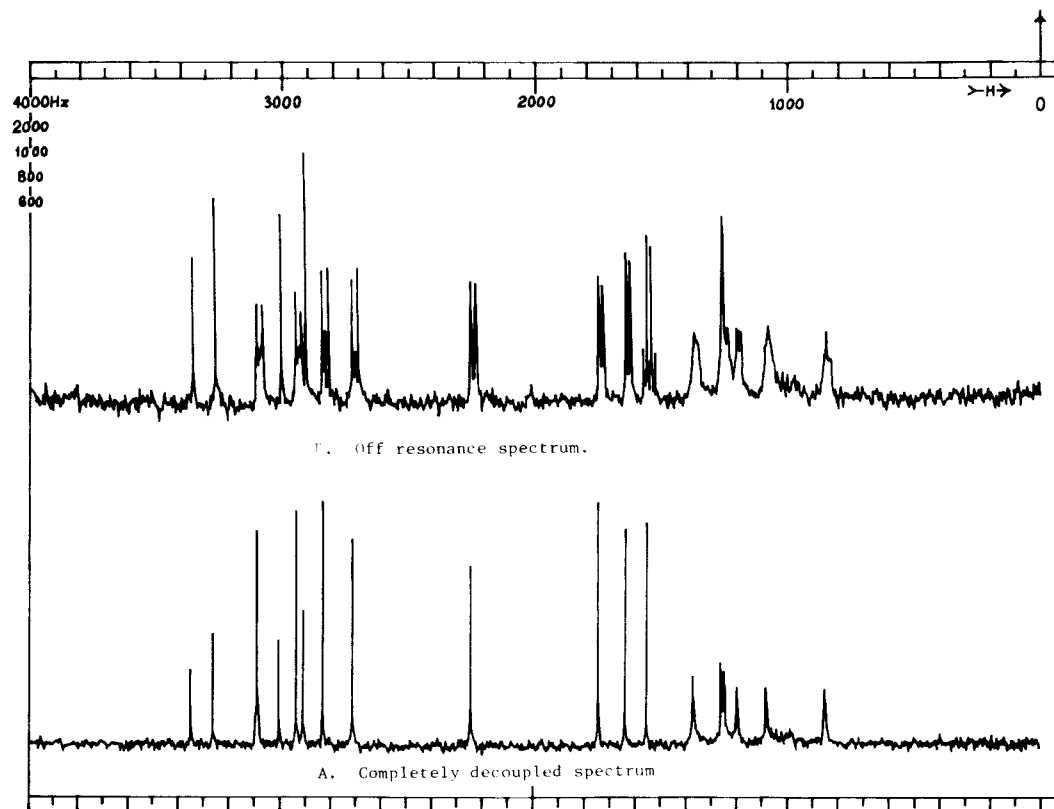


Fig. 6: ^{13}C NMR spectrum of codeine phosphate.

additivity principals and off resonance splitting pattern (Table 6).

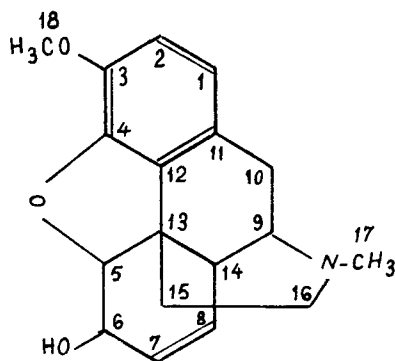


Table 6: Carbon chemical shifts of codeine phosphate.

Carbon No.	Chemical Shift ppm.		Carbon No.	Chemical Shift ppm.	
C-1	121.1	d	C-10	33.4	t
C-2	115.1	d	C-11	125.0	s
C-3	142.7	s	C-12	129.9	s
C-4	147.2	s	C-13	42.4	s
C-5	66.7	d	C-14	41.90	s
C-6	91.6	d	C-15	21.8	t
C-7	126.4	d	C-16	47.9	t
C-8	134.1	d	C-17	39.2	q
C-9	61.3	d	C-18	57.2	q

s = singlet ; d = doublet, t = triplet, q = quartet.

Carbons 3, 4, 11, 12 and 13 chemical shifts were assigned, based on relaxation data of the quaternary carbons (17).

2.5.4 Mass Spectrum

The mass spectrum of codeine phosphate obtained by electron impact ionization which was recorded on Ribermag R-10-10 mass spectrometer equipped with

direct inlet probe. The spectrum (Fig. 7) shows a molecular ion peak M^+ at m/e 299 with a relative intensity 100%.

The most prominent fragments and their relative intensities are listed in Table 7.

Table 7: Mass fragments of codeine phosphate.

<u>m/e</u>	<u>Relative Intensity %</u>
299	100 (base peak)
298	15
229	20
214	10
188	10
162	35
124	20
115	15
81	10
70	10
59	15

3. Preparation

3.1. Isolation of Codeine

Codeine occurs in opium which is the dried latex obtained from the unripe capsules of *Papaver somniferum* Linn. (Family Papaveraceae). Opium contains about 2% of codeine.

Several methods have been used for the isolation of codeine from opium. One of these is as follows:

Powdered opium is extracted with warm water to complete exhaustion. The extract is concentrated under vacuum, treated with a solution of calcium chloride (1 : 1), left for 48 hours and then filtered. The filtrate containing the hydrochlorides of the alkaloids is concentrated when morphine and codeine hydrochlorides deposit in the form of double compound known as "Gregory salt". This salt is dissolved in warm water and neutralized with dilute

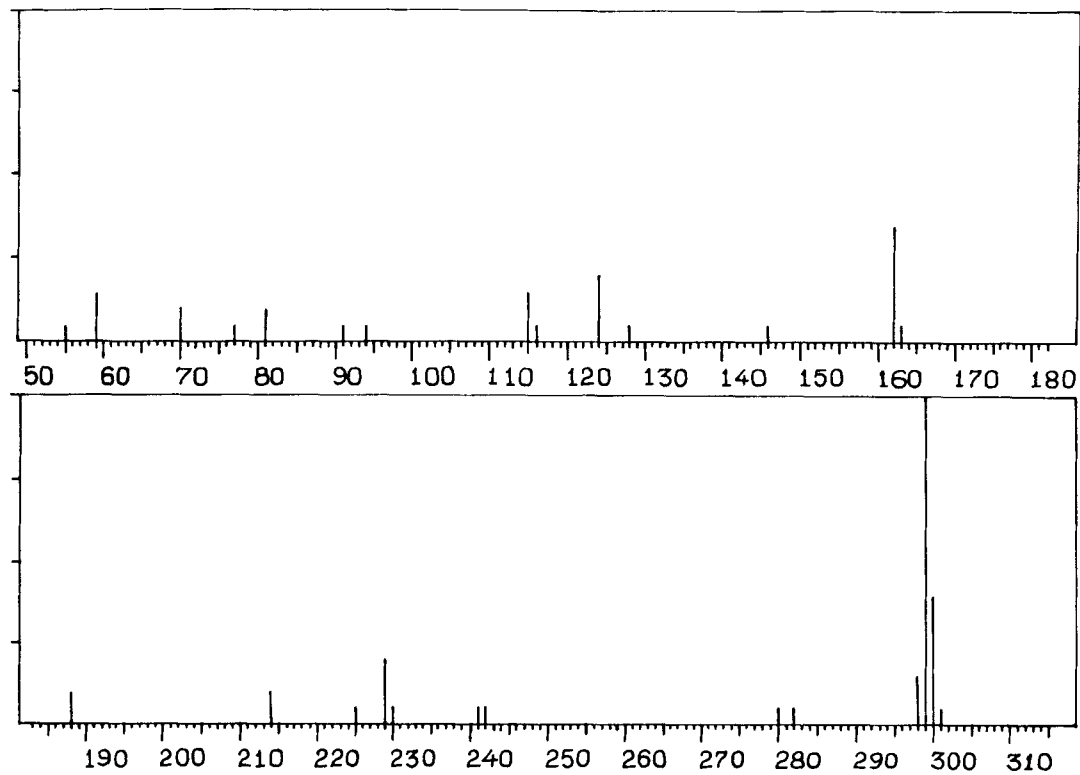


Fig. 7. The mass spectrum of codeine phosphate.

ammonia to phenolphthalein end point (pH 9). Morphine precipitates, while codeine remains in solution as ammonium-codeine chloride. The solution is concentrated, treated with 30% sodium hydroxide and codeine is then extracted with chloroform.

For further purification of codeine, the chloroformic layer is extracted with dilute sulfuric acid and the aqueous layer is decolorized with charcoal and filtered. The filtrate is rendered alkaline with sodium hydroxide solution and extracted with benzene, which is evaporated to dryness to afford codeine.

3.2. Formation of Codeine Phosphate:

This is formed by neutralizing codeine with phosphoric acid and precipitating the salt from aqueous solution with alcohol (18).

4. Synthesis of Codeine Phosphate

The first synthesis of the skeleton of the morphine alkaloids was achieved by Grewe et al (19), whose method was in fact a version of the biogenetic approach and involved an acid catalysed cyclization of benzylhexahydroisoquinoline to a morphine derivative.

4.1 Total Synthesis of Codeine (Scheme 1)

The first total synthesis of codeine was achieved in 1952 by Gates and Tschudi (2). The key intermediate "4-cyanomethyl-1,2-naphthoquinone" [8] was reacted with butadiene (Diels-Alder reaction) to give [9] which when reduced with copper chromite underwent ring closure to the ketolactam [10]. Reduction of [10] (Wolf-Kischner method) gave [11] which was N-methylated to [12] and reduced to $(\pm)\text{-}\beta\text{-}\Delta^6\text{-dihydrodesoxy codeine}$ [13]. Resolution was effected with dibenzoyltartaric acid to give the (+)-base. The resolved (+)-base was hydrated with dilute sulfuric acid to [14], followed by partial demethylation and oxidation to [15]. This was brominated to [16] and isomerized to the more stable 1-bromothebainone [17]. Reduction of [17] gave dihydrothebainone [18]. Finally, [18] was brominated and treated with 2,4-dinitrophenylhydrazine when oxide ring closure occurred to give the hydrazone [19], which upon heating with pyridine and splitting with acid gave 1-bromo-

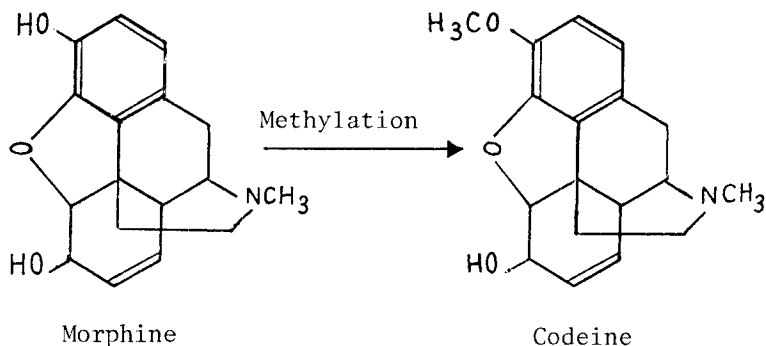
codeinone [20]. Reductive removal of 1-bromo gave codeine [21].

A rather different approach was adopted by Elad and Ginsberg (20) who synthesized (-)-dihydrothebainone. This constitutes a formal total synthesis of codeine since (-)-dihydrothebainone is transformed into codeine by Gates and Tschudi (2).

4.2 Partial Synthesis of Codeine

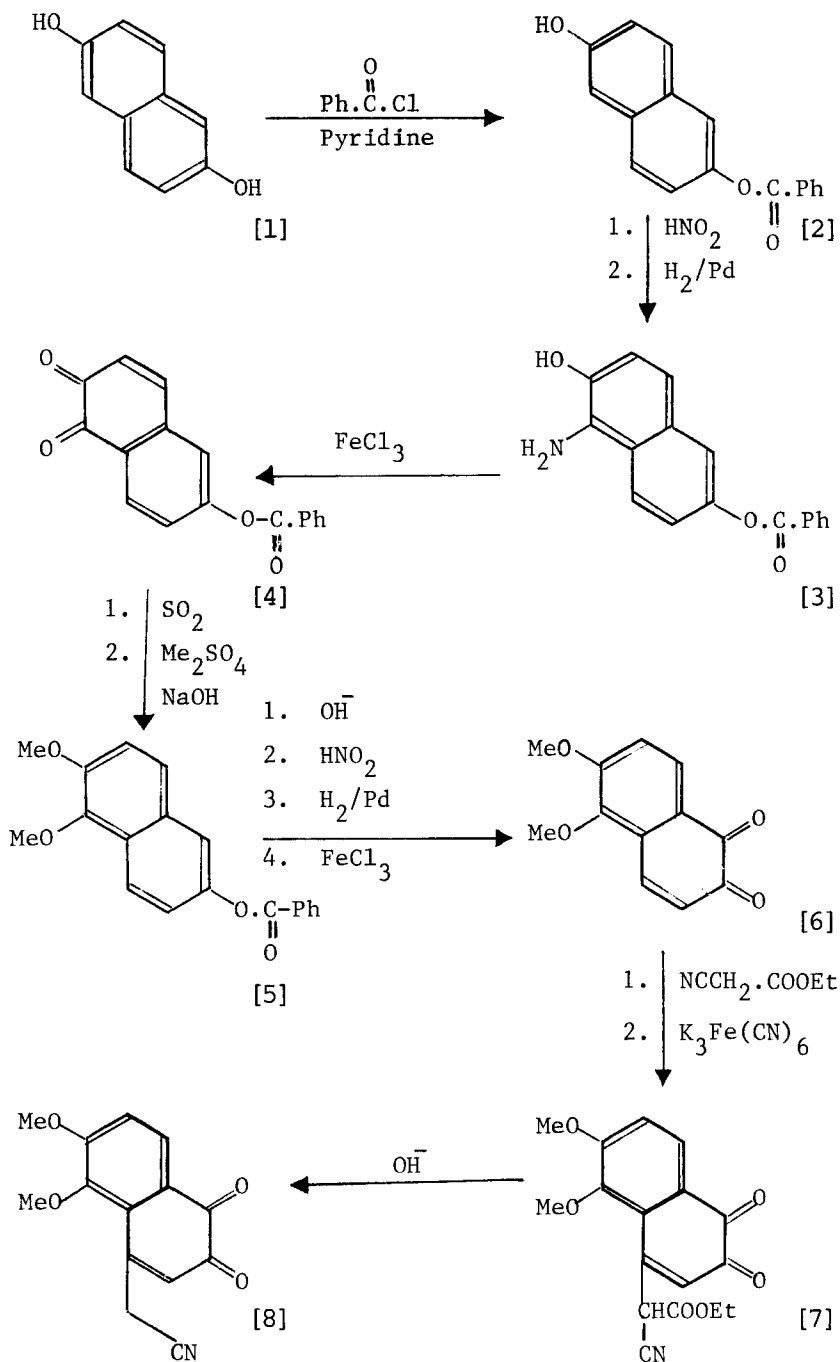
Codeine can be prepared by partial synthesis from morphine.

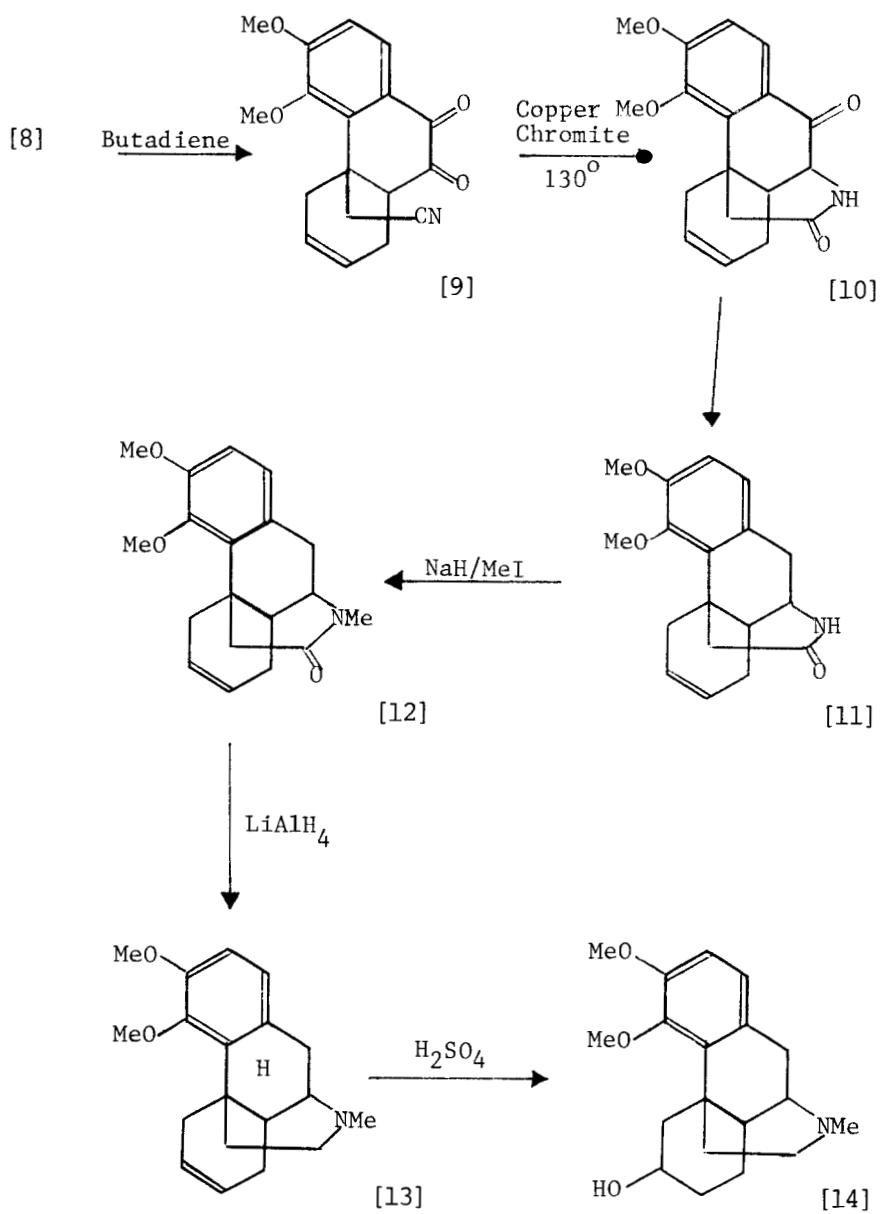
Morphine is dissolved in theoretical amount of potassium hydroxide dissolved in absolute alcohol, the required quantity of the methylating agent (usually phenyltrimethylammonium hydroxide) added, and the solution is heated at about 130°. After cooling, water is added, the solution is acidified with sulfuric acid, the dimethylaniline formed is separated, and the alcohol is removed by distillation. Treatment with caustic soda solution precipitates the codeine, while any unreacted morphine is held in solution by the sodium hydroxide. The crude codeine is purified by crystallization as the sulfate (21).

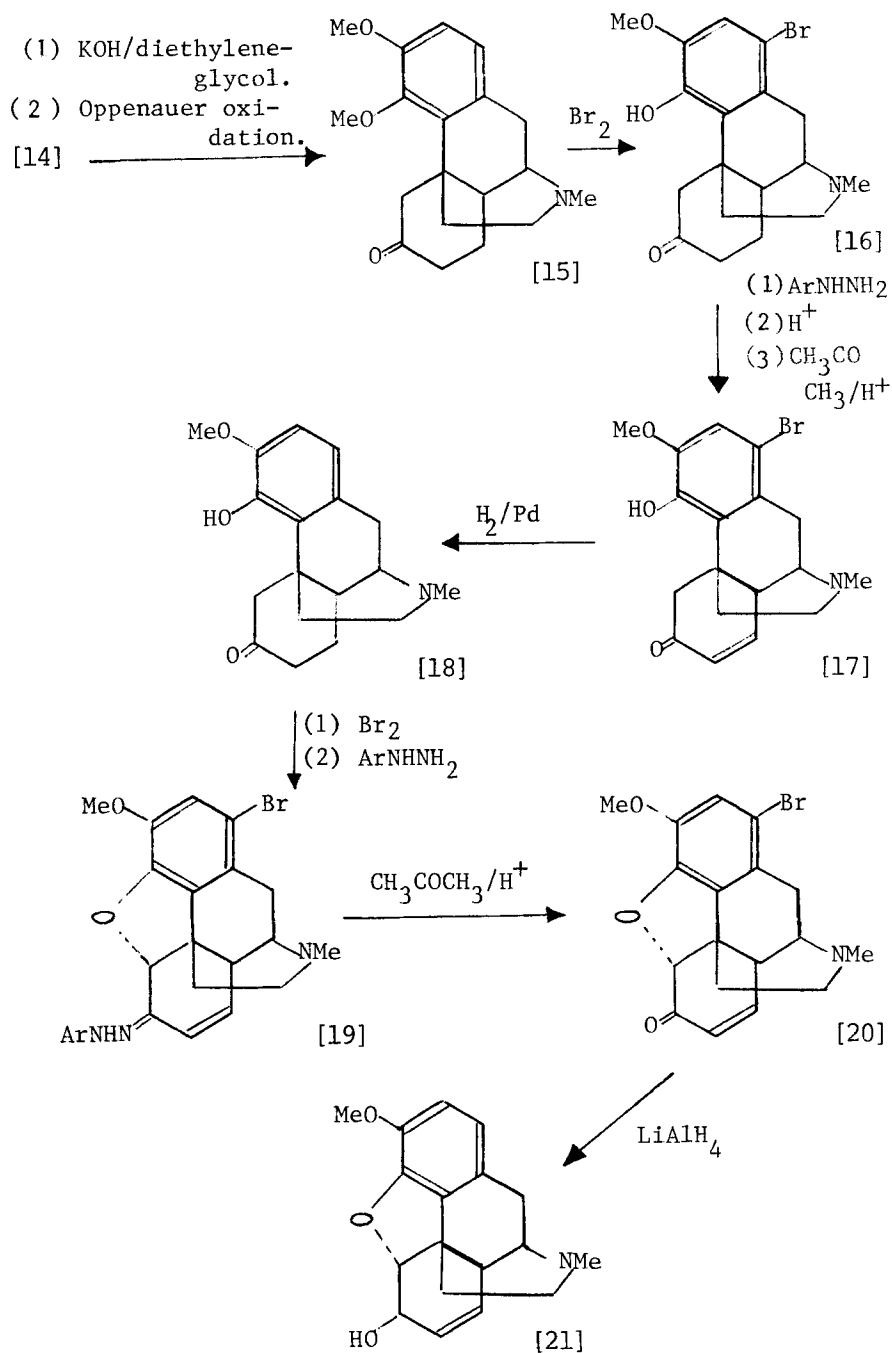


Codeine is also prepared from thebaine by appropriate reduction and demethylation (22).

Scheme 1: Total Synthesis of Codeine.







5. Biosynthesis of Codeine

Postulation of the biosynthetic pathway of opium alkaloids started in 1910 with the suggestion of Winterstein and Trier (23) that the benzyloquinoline alkaloids were built up in nature from two units of 3,4-dihydroxyphenylalanine (DOPA). These molecules, will give rise to 3,4-dihydroxyphenylethylamine and 3,4-dihydroxyphenylacetaldehyde by under going decarboxylation and oxidative deamination respectively.

Gulland and Robinson (1) proposed that morphine arises in the plant from a suitable benzyloquinoline precursor (norlaunosoline) by rotation of this precursor followed by oxidative ring closure.

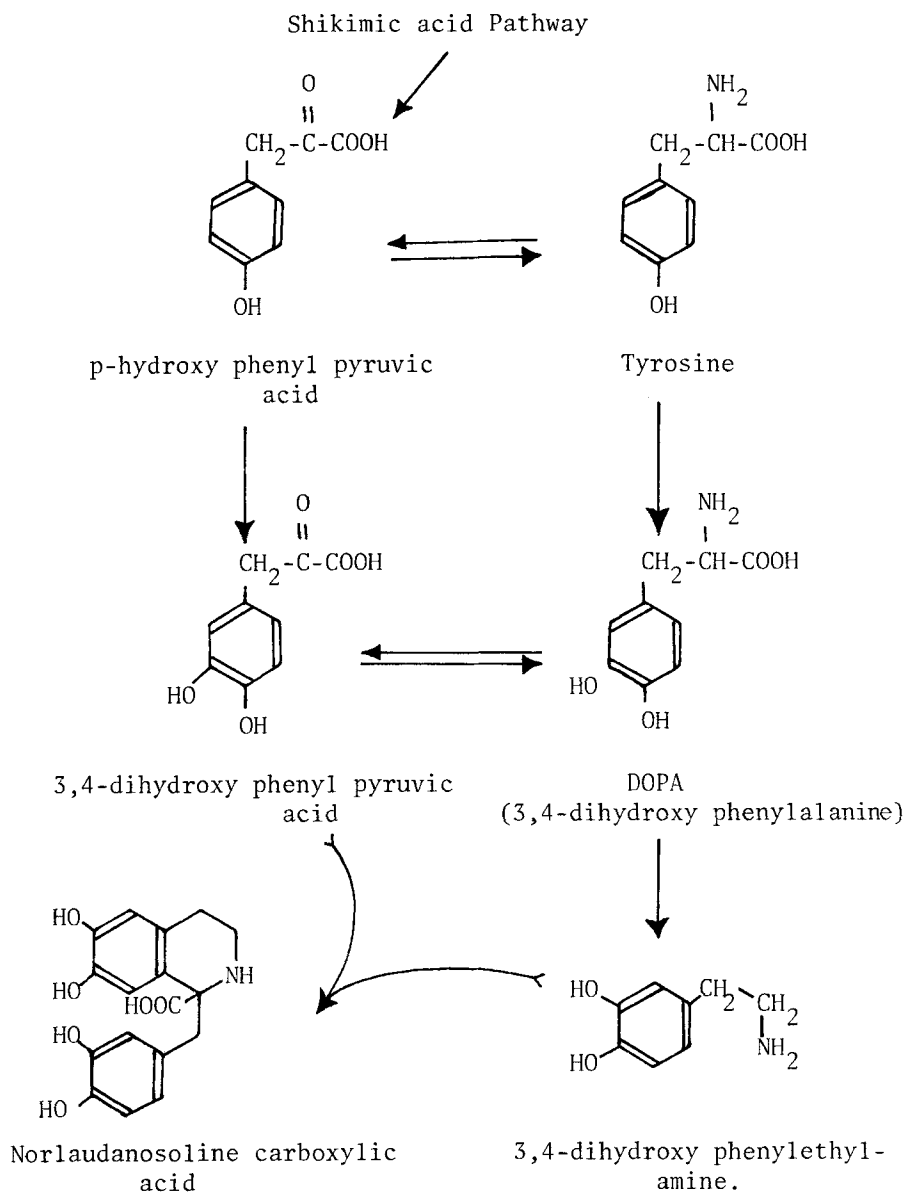
The validity of such schemes remained untested until the advent of radiochemical techniques, when in 1958 - 1960 experiments with labelled tyrosine administered to poppy capsules demonstrated that the biosynthetic routes proposed above do in fact take place in living plants.

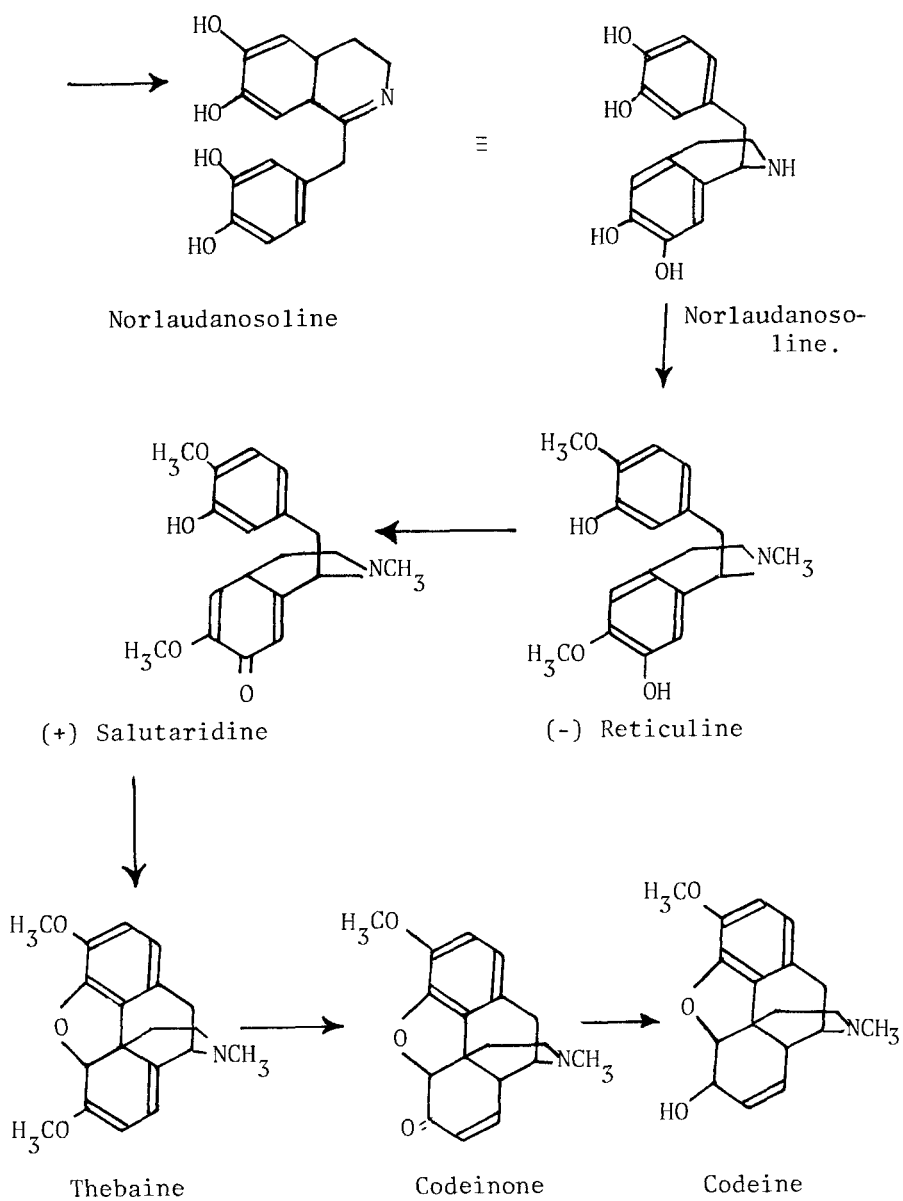
Battersby and Co-workers in 1958 and 1961 (24, 25) and Leete (26) established that when 2- ^{14}C tyrosine was fed to poppy plant, radioactive thebaine, codeine and morphine were obtained. These alkaloids were shown to be labelled equally and specifically at carbons 9 and 16 as expected. By feeding 1- ^{14}C dopamine, Battersby and Francis (27) found that only C-16 of the alkaloids was labelled but not C-9. Furthermore, Battersby et al (28) have shown that 1- ^{14}C norlaunosoline was incorporated into morphine molecule by plant with labelling at C-9 only.

These results indicated that the phenanthrene alkaloids were labelled at the anticipated sites.

Battersby et al (29) have further found that (-)-reticuline is the most efficient precursor to morphine skeleton, and this alkaloid was found to be present in opium (30). Barton et al (31) established that salutaridin which do exist in trace amounts in opium, is formed by phenolic oxidative coupling of (-)-reticuline. The existence of codeinone as an intermediate between thebaine and codeine was confirmed (32). It was suggested by Barton and Cohen (33) and Bentley and Cardwell (8) that thebaine is formed first in the plant and that codeine, then morphine arised from it. Rapaport (34, 35) has shown

Scheme 2: Biosynthesis of Codeine





by exposing *Papaver somniferum* plants to $^{14}\text{CO}_2$ for varying length of time, that radioactive thebaine was first formed in the plant and was converted into radioactive codeine and this was converted into radioactive morphine but not into thebaine. Battersby (36) has independently reached the same conclusion.

The biosynthesis of codeine is illustrated in scheme 2.

6. Metabolism

The absorption of codeine is relatively rapid after parenteral administration and erratic after oral medication (37). Codeine is metabolised mainly in the liver. The predominant metabolic changes are N-demethylation to norcodeine, O-demethylation to morphine and conjugation with glucuronic acid at the 6-hydroxyl to 6-O-glucuronide.

Experiments with either O-methyl or N-methyl ^{14}C -labelled codeine have shown that rapid disposal occurs in man and that 24 hours after injection, the maximum of morphine (4-13%), norcodeine (8%), bound codeine (35 - 40%) and unchanged codeine (5 - 12%) is present in the urine ; negligible amounts are found in the feces and only part of the detached O-methyl and N-methyl radicals can be recovered as expired carbon dioxide (38, 39).

In 24 hours following oral administration of 4 mg/Kg in divided doses, 4% of the dose was excreted in the urine as free norcodeine, 14% as conjugated norcodeine, 1% as free morphine and 7% as conjugated morphine (16).

The metabolism of codeine is presented in scheme 3.

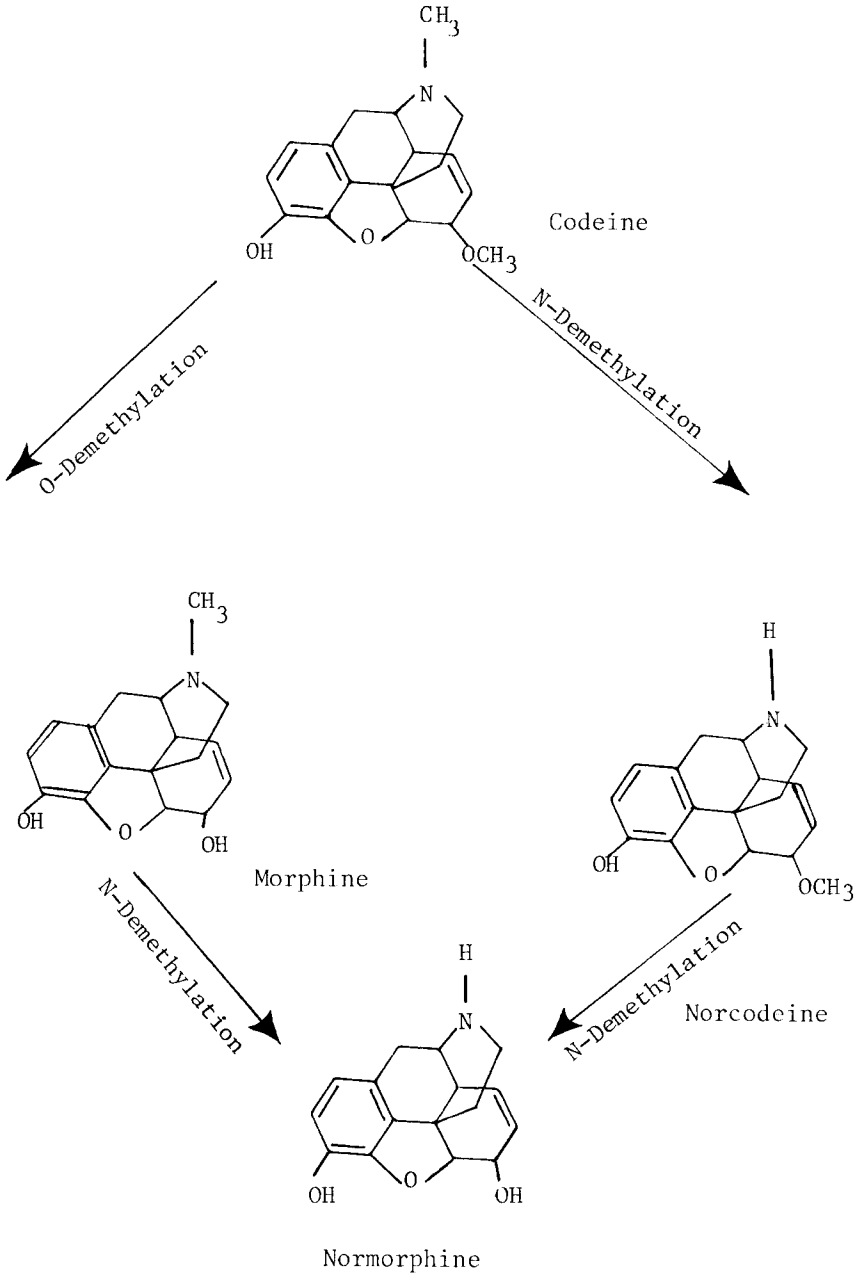
7. Methods of Analysis

7.1 Identification Tests

The following identification tests are those mentioned in the U.S.P.XX(11):-

- A) The infrared absorption spectrum of a potassium bromide dispersion of it, previously dried, exhibits maxima only at the same wavelengths as that of a similar preparation of USP Codeine Phosphate Reference Standard.

Scheme 3: Metabolism of Codeine.



- B) Dissolve 100 mg in 15 ml of water. Render the solution alkaline with ammonia TS, extract with three 5-ml portions of chloroform, filter the combined chloroform extracts through filter paper that previously has been washed and moistened with chloroform, and evaporate the combined chloroform extracts on a steam bath just to the disappearance of the chloroform odor: the residue of codeine melts between 154° and 158° .
- C) To 1 mg contained in a porcelain crucible or small dish add 1 drop of sulfuric acid containing, in each ml, 5 mg of selenious acid: a green color is produced at once, and it rapidly changes to blue, then slowly to dark olive-green.
- D) To a solution of 5 mg in 5 ml of sulfuric acid contained in a test tube add 1 drop of ferric chloride TS, mix, and heat in boiling water for 2 minutes: a blue color is produced and upon the addition of 1 drop of nitric acid changes to red-brown.
- E) Neutralize a solution (1 in 50) with ammonia TS, and add silver nitrate TS: a yellow precipitate of silver phosphate is formed, and it is soluble in diluted nitric acid and in ammonia TS.

Other identification tests (16, 40) are as follows:

Place a little in powder, on the surface of a drop of nitric acid, a yellow color is produced.

Add to a little of codeine, 1 ml of sulfuric acid containing drops of formaldehyde, a purple color is formed (sensitivity $0.05 \mu\text{g}$).

Add drops of ammonium molybdate to codeine phosphate, a green color is produced (sensitivity $0.1 \mu\text{g}$).

7.2 Microcrystal Tests

Add potassium cadmium iodide solution to codeine

phosphate, gelatinous rosettes crystals are formed, changing to aggregates of small tablets (16).

Add potassium tri-iodide solution to codeine, feathery rosettes crystals formed overnight (16).

7.3 Titrimetric Methods

The official methods of determining codeine phosphate are described by the B.P. (40) and U.S.P.XX(11).

7.3.1 Aqueous Titration

The B.P. describes a method for assaying codeine phosphate in tablets as follows:-

Weigh and powder 20 tablets. Dissolve a quantity of the powder equivalent to 0.3 g of Codeine Phosphate as completely as possible in 20 ml of 0.5 N sulfuric acid, filter, with 0.5N sulfuric acid until complete extraction of the alkaloid is effected. Make alkaline with dilute ammonia solution, and extract with successive quantities of chloroform until complete extraction of the alkaloid is effected. Wash each chloroform solution with the same 10 ml of water. Evaporate the chloroform. To the residue add 5 ml of alcohol (95 per cent) previously neutralised to methyl red solution and remove the alcohol by evaporation. Dissolve the residue in 1 ml of neutralised alcohol (95 per cent), add 10 ml of 0.1N hydrochloric acid and 10 ml of water, and titrate with 0.1N sodium hydroxide, using methyl red solution as indicator. Each ml of 0.1N hydrochloric acid is equivalent to 0.04064 g of $C_{18}H_{21}NO_3 \cdot H_3PO_4 \cdot \frac{1}{2}H_2O$.

Other methods have been also reported utilizing 0.01 M aqueous sodium dioctylsulfosuccinate as a titrant using dimethyl yellow (41), or a mixture of 0.1% dimethyl yellow and 0.1% malachite green solution in chloroform (42) as indicators.

7.3.2 Non-Aqueous Titration

The U.S.P. (XX) describes the following method:-

Dissolve about 1 g of Codeine Phosphate, accurately weighed, in 20 ml of glacial acetic acid, warming slightly if necessary to effect solution, and titrate with 0.1 N perchloric acid, determining the end-point potentiometrically. Perform a blank determination and make any necessary correction. Each ml of 0.1 N perchloric acid is equivalent to 39.74 mg of $C_{18}H_{21}NO_3 \cdot H_3PO_4$.

Another method is as follows (43):-

Codeine phosphate is treated with NaOH or Na_2CO_3 extracting the liberating base into $CHCl_3$ evaporating most of the solvent and titrating with $HClO_4$ in dioxan using dimethyl yellow and methylene blue as indicator.

A third method for micro-determination of codeine phosphate in tablets is described (44). 60 mg of codeine phosphate is dissolved in water, render alkaline with sodium hydroxide solution and extracted with ethanol-free chloroform, filter the extract through cotton wool and anhydrous sodium sulfate into a 50 ml flask and dilute with chloroform to the mark. To an aliquot of 10 ml of codeine solution, add 0.1% dimethyl yellow solution in chloroform and titrate with 0.005 N toluene-para-sulfonic acid in chloroform and carry out a blank determination.

7.4 Complexometry

- a) Codeine phosphate is dissolved in water, ethanol and aqueous NaOH are added. To the resulting mixture add 0.2 M copper picrate (30 ml), mix and after 30 min. collect the precipitate on a sintered-glass filter (G4) and wash with water (5 x 5 ml). To the combined filtrate and washings add 0.2 M EDTA (20 ml) and ammonia buffer solution of pH 10.4 (0.3 ml) and murexide-NaCl

(1 : 200) and titrate the excess of EDTA with 0.2 M ZnSO_4 until the solution is green (45).

- b) Excess of Standard MgSO_4 solution is added to the hot slightly ammonical codeine phosphate solution, which (after filtering off the precipitated MgNH_4PO_4) is back titrated with EDTA (disodium salt) using Eriochrom black T as indicator (46).

7.5 Spectrophotometry

7.5.1 Colorimetry

A colorimetric procedure was described for the assay of codeine phosphate using picric acid (47). An aqueous solution (1.0 ml) containing about 0.5 mg of codeine phosphate is treated with 0.5 ml buffer solution (300 g in $\text{Na}_2\text{H}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and 9 g of NaOH made up to 750 ml with water), 1.0 ml of picric acid solution and 10 ml of chloroform (alcohol free). The mixture is shaken for 30 seconds and the extinction of the filtered chloroform phase is measured at 430 nm. A calibration curve is constructed from readings obtained with standard preparations.

Another method is based on the reaction with bromothymol blue (48). Codeine phosphate is dissolved in 0.5% HCl (v/v) and treated with a 0.04% solution of bromothymol blue using McIlvaine buffer solution of pH 8. The sample is then extracted with chloroform and the extinction of the extract is measured at 410 nm.

A third method involving the formation of molybdophosphoric acid from codeine phosphate with a mixture of H_2SO_4 and HNO_3 . Chromethylpyrazol is added and the extinction of the resulting color is measured at 620 nm. (49). A calibration curve is prepared using 0.5 to 3.5 ml of 64.5 (M- KH_2PO_4 add 0.5 ml of 5% ammonium molybdate solution, 3 ml of 2 M HNO_3 , 1 ml of 1% starch solution and (after 3 to 4 minutes) 3 ml of 0.4 mM. of chromethylpyrazol, dilute each solution to 25 ml, warm at 30° for

40 minutes, and measure the absorbance at 620 nm.

7.5.2 UV

A stability indicating assay has been reported (50) for the determination of codeine phosphate in syrups. The syrups were subjected to accelerating ageing at 60°, 70° and 80° and the content of codeine phosphate was then determined spectrophotometrically at 285 nm, after separation of codeine phosphate by chromatography on Whatman No. 3MM paper impregnated with 0.5 M-KH₂PO₄, the developing solvent is isobutyl alcohol/water/ethanol/acetone/ethylacetate (8:5:2:2:1).

Another method was reported for the determination of codeine phosphate in mixtures (51), with mean recoveries in the range 98.5 to 99.85%. The powdered sample is mixed with water and diluted to 50 ml, then centrifuged and applied as 20 ml portions to a column (35 x 2 cm) of alginic acid. Elute codeine phosphate with 0.01 N HCl and measure the extinction at 250 nm and 273 nm.

7.5.3 NMR

A rapid, accurate and precise PMR method is reported (52) for the quantitative determination of codeine and codeine phosphate as bulk drugs and in tablet dosage form. The determination is based on the integration of the C-3 methyprotons or the two aromatic protons of codeine or its salt relative to that of the nine protons of t-butanol. Standard deviations of ± 1.39 , 0.27 and 0.65% were obtained for codeine, codeine phosphate bulk drug and codeine phosphate tablets respectively. The sample powder (or powdered tablets is mixed with 2 ml of the internal standard soln. (10 mg ml⁻¹ of t-butyl alcohol in ethanol-free chloroform for codeine or in water for codeine phosphate), with shaking for 3 min. After centrifuging the mixture, 0.5 ml of the clear soln. is subjected to n.m.r. at 60 MHz. Repeated integrations are

made of the peaks at 3.8 and 1.23 p.p.m. (corresponding to the C-3 methyl protons of codeine and the nine protons of t-butyl alcohol) for codeine and of those at 6.83 and 1.27 p.p.m. (two aromatic protons of codeine phosphate; nine protons of the alcohol) for codeine phosphate. No interference is observed from excipients in the tablets. The detection of morphine in codeine and its salts is possible, as the aromatic-proton doublet for morphine is at 6.7 p.p.m.

7.5.4. Mass

Opium alkaloids can be determined from their mass spectra by comparison of the parent peak of each individual alkaloid with that of a reference compound added in known amount (53, 54) and by reference to a calibration graph. At a low ionisation voltage, the M^+ ion can be detected clearly, but the peak is small. At higher ionisation voltages, the M^+ ion peak becomes higher, but many other fragment peaks overlap it. The relationship between the intensity of the M^+ ion peak and ionisation voltage was studied, and appropriate ionisation voltages were determined.

Tatematsu et al (55), have found that the mass spectra of codeine salts (hydrochloride, hydrobromide, sulfate, phosphate, oxalate, malonate, succinate, tartarate, citrate, meconate and picrate) showed the spectrum of the free base (codeine) with an M^+ peak at m/e 299. The relative intensity of the main fragment was the same for all salts. It was considered that the temperature at which the M^+ ion peak appeared was related to volatility and the acid radical and the height of the M^+ ion peak to the strength of the acid.

7.6 Chromatography

7.6.1 Paper Chromatography

a) Qualitative Paper Chromatography

The R_f values of codeine in different

solvent systems are listed in table 8.

Table 8: Rf values of codeine on paper chromatography.

Chromatogram	Solvent	Rf value of codeine	Location Reagent
1. Whatman No. 1, buffered by dipping in 5% solution of sodium dihydrogen citrate, blotting and drying at 25° for 1 hour (16).	4.8 g of citric acid in a mixture of 130 water & 870 ml of n-butanol	0.16	- Examination under UV (254 mu). - Iodoplatinate reagent - Dragendorff reagent
2. Reversed phase chromatography. Whatman No.1 impregnated with 10% solution of tributyryl in acetone and drying in air (16).	Acetate buffer (pH 4.58) or Phosphate buffer (pH 7.4)	0.89 0.22	- Iodine vapor
3. Whatman No.1, impregnated with 0.5 M KH_2PO_4 (56) (pH 4.2)	Cyclohexane/chloroform/diethylamine (7:2:1)	0.56	

b) Quantitative Paper Chromatography

A descending technique on Whatman No. 1 paper strips. The paper is impregnated with a solution of ammonium sulfate (2%). Freshly prepared isobutanol/acetic acid/water (10:1:2.4), is used

as solvent (56). The aqueous solution of codine phosphate (or ethanolic solution of the alkaloid) is spotted by means of an "Alga" micrometer syringes. Five microliteres, which should contain 5-50 μg of the alkaloid is applied, yielding a spot not larger than 5 mm in diameter. The paper is equilibrated for six hours in a jar which is saturated with solvent vapors.

Chromatography takes place overnight (16 hours), in which time the solvent front travels about 38 cm. After drying the chromatogram is sprayed on both sides, thoroughly and uniformly with potassium iodoplatinate reagent and dried again for 15 min. in a current of air. The total color density of the blue spots on white background are scanned directly by utilizing a self-integrating densitometer.

The standard curve of codine phosphate is prepared by plotting the concentrations (in μg .vs. total density of the spot).

7.6.2 Thin Layer Chromatography (TLC)

The Rf values of codeine phosphate in different solvent systems are listed in Table 9.

Table 9: Rf values of Codeine Phosphate.

Chromatograms	Solvent System	Rf value of codeine	Location Reagent
1. Silica gel G	Methanol/strong ammonia solu- tion (100 : 1.5)	0.35 (16)	- Acideified iodoplati- nate spray - Dragendorff spray - Examination under

2. Silica gel G	Benzene/dioxan/ ethanol/strong ammonia solution (50:40:5:5)	0.39 (16)	- U.V. (254 nm.)
3. Silica gel G	Acetic acid/ ethanol/water (30:60:10)	0.29 (16)	
4. Silica gel G	Xylene/ethyl- methylketone/ methanol/di ethylamine (20:20:3:1)	0.30 (57)	
5. Silica gel G	Xylene/acetone/ methanol/ ammonia 0.88 (20:20:3:1)	0.24 (58)	
6. Silica gel G	-	-	2% HgCl ₂ solution containing 0.01% methyl red (59) or 2% HgCl ₂ solution, drying in an oven and spraying with KI solution (59).

A quantitative evaluation of codeine phosphate in drug mixtures using TLC by measuring its remission and fluorescence has been reported (60). A powdered tablet containing codeine phosphate, phenobarbital, caffeine, aspirin and phenacetin is extracted with methanol/diethylamine (99:1) (2 x 5 ml) and then with dichloromethane (2 x 5 ml), and the combined extracts are diluted to 25 ml with dichloromethane. This solution is applied to a pre-coated Kieselgel G (Merck) plate, which is developed with cyclohexane-CHCl₃-

acetic acid (6:3:1) and /or acetone- CHCl_3 -25% aqueous NH_3 (65:35:4) and the re-mission spectra are measured directly on the plate.

Other TLC data have been also reported (61-64).

7.6.3 Column Chromatography

Different column chromatograms have been employed for the separation, purification and quantitation of codeine phosphate in drug mixtures (65-67).

7.6.4 Gas Chromatography (GC)

The gas chromatograms for codeine are listed in Table 9.

Table 9: The GC of Codeine.

Column Condi- tions.	Carrier Gas	Detec- tor	Standard	Retention time.
1. 2.5% SE-30 on 80-100 mesh chromosorb W (5 ft x 4 mm internal dia- meter) column temp.: 225°.	Nitrogen, 50 ml/min.	FID H_2 50 ml/ min. air 300 ml/ min.	Diphenhy- dramine	4.65 (16)
2. 3% XE-60 sili- cone nitrile polymer on 100-120 mesh chromosorb W, column temp.: 225°.	Nitrogen, 50 ml/min.	FID H_2 50 ml/ min. air 300 ml/ min.	Diphenhy- dramine	9.6 (16)
3. 3.8% of sili- cone gum (Lin- de W 98) on	Helium 55 ml/min.	FID	-	Peak height. The Coeff.

Diatoport C (80-100 mesh), column temp.: 225°.					of variation ± 4.93% (68)
4. 2% dexil 300 on varaport 30 (6 ft x 2 mm) column temp.: 280°.	Nitrogen 60 ml/min.	FID	-		Peak height or area (69).
5. 3% DV-17 on silanised chromosorb W AW (80 to 100 mesh) (100 cm x 0.64 cm o.d.) column temp.: 240°.	Helium 28 ml/min	FID	-		(70)
6. 3% OV-17 on gas chrom Q (100 - 120 mesh) column (1.8 m x 2 mm), column temp. : 120°.	Nitrogen 35 ml/min.	FID	Propyl-4- hydroxy- benzoate		Coefficient of vari- tion ± 0.42% for codeine phosphate and acetyl codeine phosphate (71).

7.6.5 High Performance Liquid Chromatography (HPLC)

Codeine phosphate was determined in a cough syrup by high-pressure liquid chromatography (72) as follows:-
The cough syrup is diluted with 0.05 M- KH_2PO_4 solution in aqueous 13% methanol and determined directly by HPLC on a Bondapak- C_{18} column (30 cm x 4 mm) with KH_2PO_4 solution as mobile phase (2 ml/min) and detection at 254 nm.

A second method is employed for the

separation of opium alkaloids by high-performance liquid chromatography, on Partisil, with methanol/2N-NH₃/1N NH₄NO₃ (30:2:1) as mobile phase or on Silica RP-18 with acetonitrile/0.01N -(NH₄)₂CO₃ (2:3) as mobile phase. The extinction of each eluate is measured at 278 nm. Step-wise-gradient elution can be used to achieve more rapid elution of the slower-migrating contaminants (73).

A third method is also reported in 1980 (74) for the separation and determination of opium alkaloids by HPLC as follows:

A rapid and simple method for the routine determination of morphine, codeine, cryptopine, thebaine, narcotine and papaverine in gum opium is used. The sample is extracted with 2.5% acetic acid and an aliquot of the extract is diluted threefold with methanol before analysis on Nucleosil-10CN with 1% ammonium acetate buffer solution (adjusted to pH 5.8 with acetic acid)/acetonitrile/dioxan (8:1:1) as mobile phase (1.5 ml/min.) detection is at 254 nm. For a sample of gum opium from India, the coeff. of variation (16 results for each alkaloid) were <1.5% ; the cryptopine content was below the detection limit.

A stability indicating assay for codeine phosphate by utilizing HPLC has been reported (75). The best results were obtained by HPLC on a column (30 cm x 4 mm) of Bondapak -C₁₈, with aqueous 0.1 M-KH₂PO₄ /methanol (21 : 19) as mobile phase (21 ml/min.) and detection at 254 nm.

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COLCHICINE

Dorothy K. Wyatt, Lee T. Grady, and Sy-rong Sun

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1. History

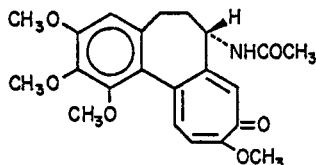
Colchicine in impure form (*Colchicum*) has been known to man for thousands of years [1]. It is the active ingredient of one of eighteen plants still in use of the approximately 700 listed in the Ebers Papyrus of ancient Egypt (1550 B.C.). Dioscorides [23,1], Nero's personal physician, provided the earliest remaining complete botanical description of *Colchicum autumnale*, the autumn crocus or meadow saffron, whose seeds, powdered corm, and dried flowers contain sufficient colchicine to effect relief of pain.

Colchicum use for the treatment of gout was documented in approximately 560 A.D. and use appears to have been widespread until the eleventh century. Although relief of pain was obtained quickly, its high toxicity led to disuse (see #9). British formularies did list, then discard, colchicum in the early 1600's (London Pharmacopoeia and Complete English Dispensatory) but it was not until the early 1800's that colchicine use again became widely established. Colchicine's other biological characteristics, namely, its highly specific association with microtubule proteins and its effects on basic cell functions such as mitosis, secretion, cell morphology, motility, intracellular transport of macromolecules, microtubular assembly, and mitogenic activation [51], make it a highly studied and widely applicable compound for use medicinally and for biochemical and biomedical research.

2. Description

2.1 Name, Formula, Molecular Weight

Colchicine is *N*-(5,6,7,9-tetrahydro-1,2,3,10-tetramethoxy-9-oxobenzo[*a*]heptalen-7-yl)-, (*S*)-acetamide. The CAS registry number is 64-86-8 [2,3].



$C_{22}H_{25}NO_6$
molecular weight 399.44

2.2 Appearance, Color, Odor

Pale yellow, amorphous scales, or powder. Is

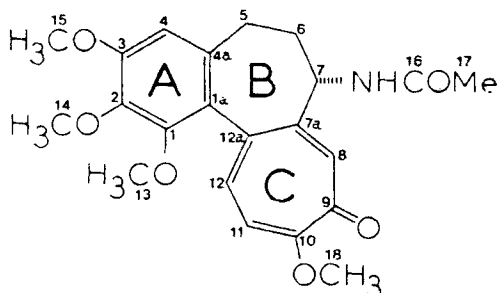
odorless or nearly so, and darkens on exposure to light [4,5,6,9,10].

3. Physical Properties

3.1 Infrared Spectrum

The infrared spectrum is presented in Figure 1. The spectrum was obtained from a potassium bromide dispersion of previously dried material (105°, 3 hours) using a Beckman 5260 grating infrared spectrophotometer. Principal bands are 1248, 1566, 1589 cm^{-1} [7].

3.2 Nuclear Magnetic Resonance Spectra



3.2.1 Proton NMR Spectrum

The proton NMR spectrum is presented in Figure 2 and spectral assignments in Table I. The spectrum was obtained using a Varian FT-80A instrument. Sample concentration: 500 mg/2.5 ml; tube diameter: 5 mm; spectral width: 1000 Hz; acquisition time: 1.023; pulse width: 60 sec.

Table I

^1H NMR Spectral Assignments for Colchicine [8]

<u>Chemical Shift*</u> <u>ppm</u>	<u>Multiplicity</u>	<u>Characteristic of Proton</u>
6.57	singlet	4-H
7.67	singlet	8-H
6.93	doublet ($J = 11$)	11-H
7.40	doublet ($J = 11$)	12-H

*Chemical shifts (δ) in CDCl_3 (TMS internal standard) at 100 MHz; J = coupling constant in Hz.

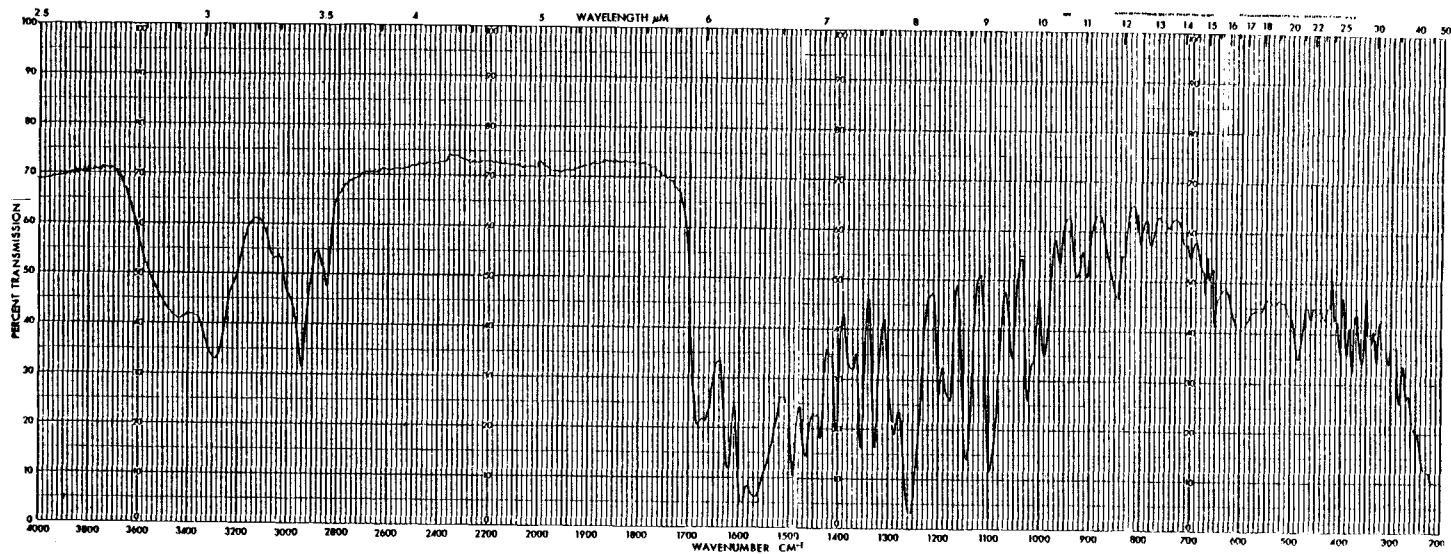


Fig. 1. Infrared spectrum of colchicine 0.5% potassium bromide dispersion.

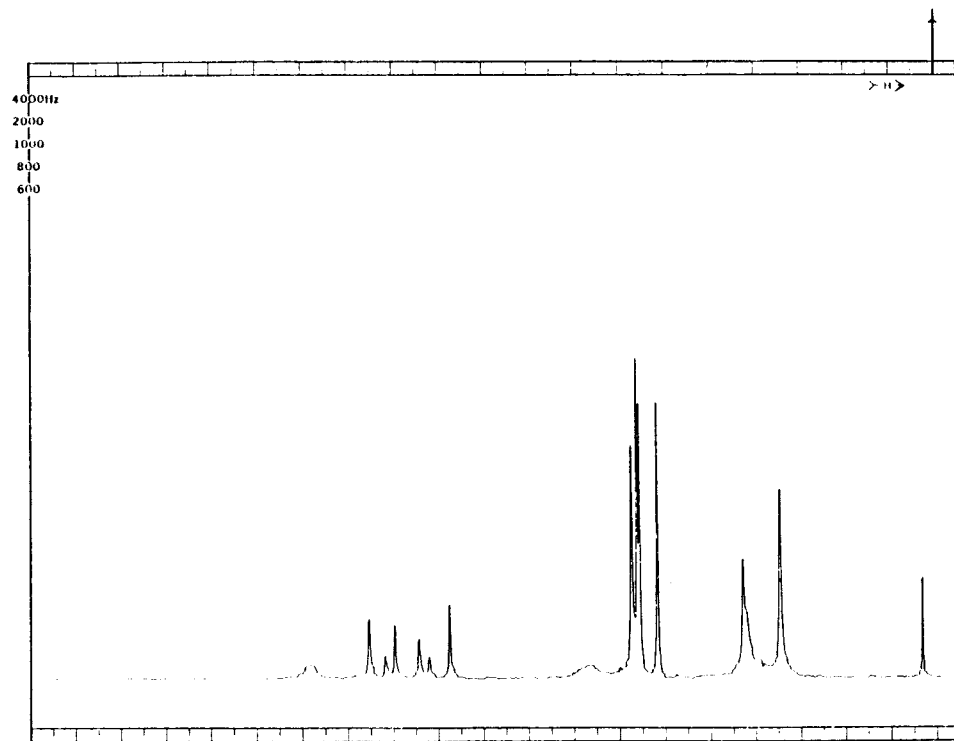


Fig. 2. Proton NMR spectrum of colchicine.

3.2.2 Carbon-13 NMR Spectrum

The carbon-13 NMR spectrum is presented in Figure 3.

Spectrometer: Varian FT-80A; sample concentration: 500 mg/2.5 ml; tube diameter: 10 mm; spectral width: 4000 Hz; acquisition time: 1.023 sec.; pulse width (flipping angle): 8 sec. (45°); number of data points: 10,230.

Spectral assignments are listed in Table II [15,16,18].

Table II

¹³C NMR Spectral Assignments for Colchicine

<u>Chemical Shift</u> (δ in ppm relative to TMS)		<u>Carbon Number</u>
60.9		13
60.7		14
56.0		15
168.9		16
22.4		17
55.9		18
125.7		Ring A { 1a
150.7		
141.1		
153.2		
108.0		Ring B { 4a
134.4		
29.4		
36.0		
51.7		Ring C { 7a
151.2		
134.7	[C12-(15,18)]	
178.4		
163.8		Ring C { 10
112.3		
130.7	[C8-(15,18)]	
135.6		

3.3 Ultraviolet Spectrum

The ultraviolet absorption spectrum of colchicine obtained from a 1 in 100,000 solution in chloroform is shown in Figure 4. One absorption maximum was observed at about 350 nm with an absorptivity of about 45 [8]. The ultra-

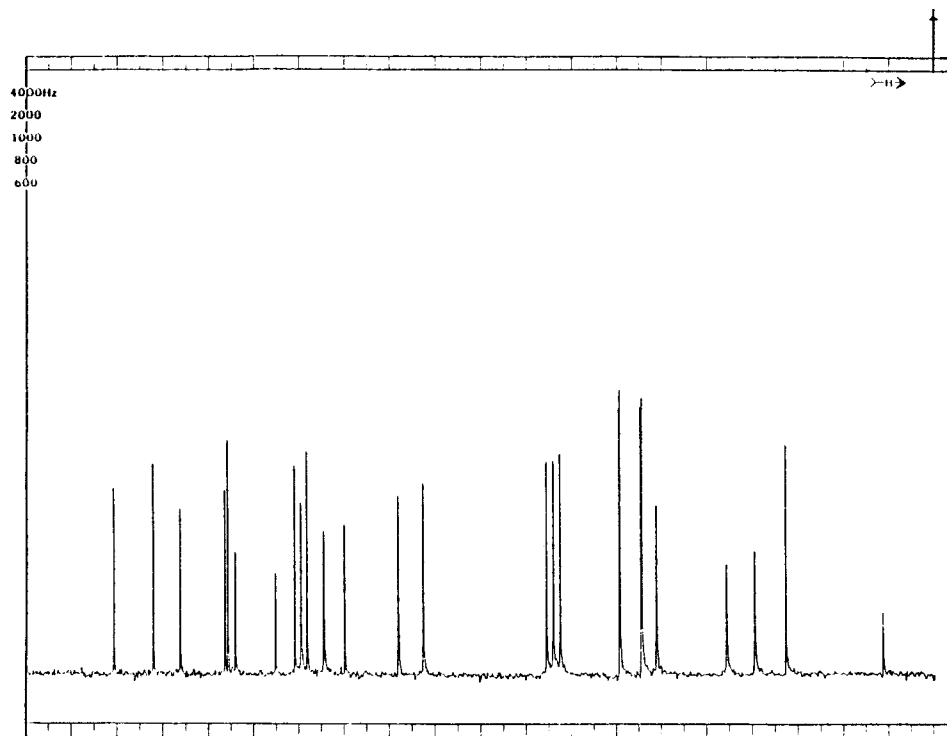


Figure 3: Carbon-13 NMR spectrum of colchicine.

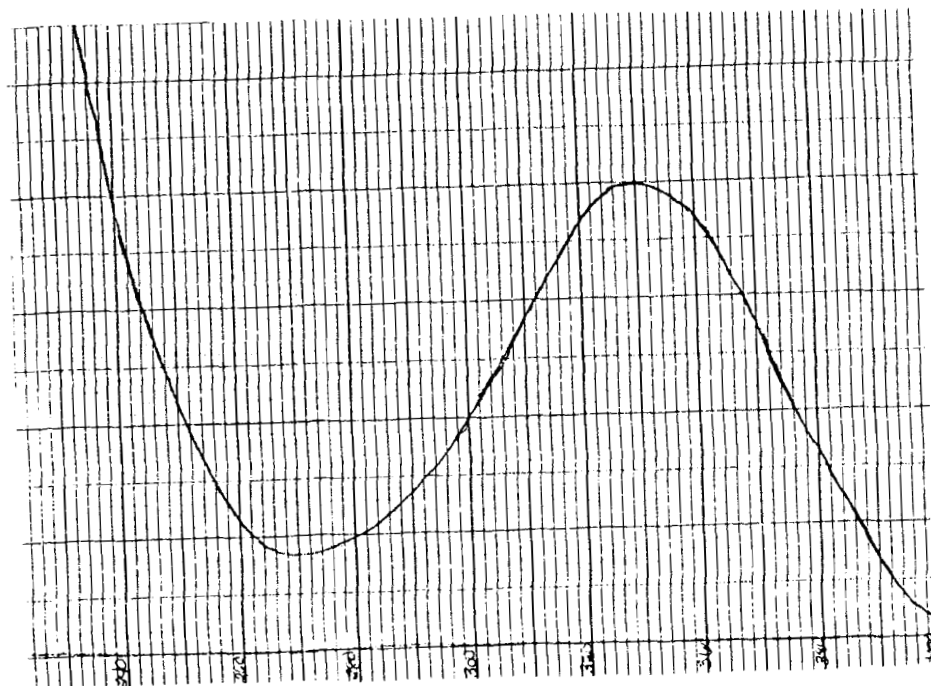


Figure 4: Ultraviolet spectrum of colchicine in chloroform

violet absorption spectrum of colchicine obtained from a 1 in 100,000 solution in 95% ethanol is shown in Figure 5 [5,8]. Two absorption maxima were observed at about 351 and 243 nm with absorptivity values of 45 and 81, respectively.

3.4 Mass Spectrum

The mass spectrum is shown in Figure 6, and the fragmentation pattern is presented in Table III and IV. The mass spectrum was measured using a CEC 21-103C mass spectrometer equipped with a "direct" sample inlet system with the isotron temperature at 270°. The ionization energy is maintained at 70 eV, the ionizing current at 50 μ A [20,40].

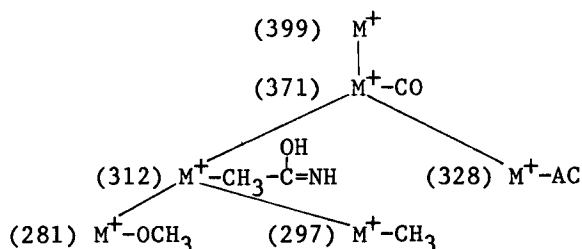
Table III

Mass Spectrum Fragmentation Pattern of Colchicine

m/e	Species
399	M^+
371	$M^+ - CO$
312	$M^+ - CH_3 - \overset{OH}{\underset{ }{C}}=NH$
297	$M^+(312) - CH_3$
281	$M^+(312) - OCH_3$

Table IV

Mass Spectrum Fragmentation Scheme for Colchicine
[(m/e), species]



3.5 Melting Range

The melting range of a colchicine sample, determined after drying at 105°C for 3 hours is between 140° and 141.5°C according to the United States Pharmacopeia XX Class I procedure [2]. Additional melting points using unspecified procedures and colchicine of unspecified purity or hydration are given in Table V.

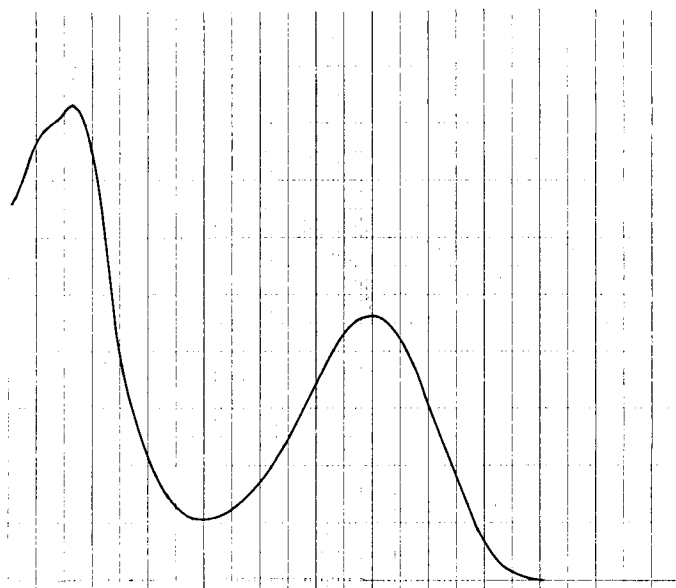


Figure 5: Ultraviolet spectrum of colchicine in 95% ethanol

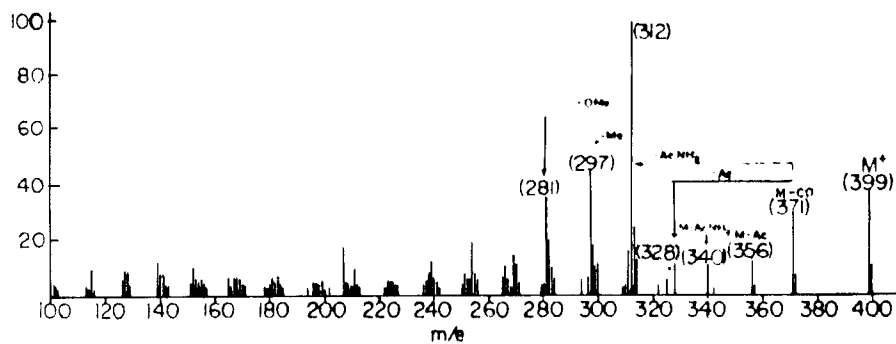


Figure 6: Mass Spectrum of Colchicine

Table V

<u>Melting Point (°C)</u>	<u>Reference</u>
142-150	5
153-157	7, 10
155-157	21
143-147	23
145	6
155	1

3.6 Solubility

The approximate solubilities obtained at room temperature are listed in Table VI [5,6,7,9,10]. Anhydrous colchicine decreases in solubility with increasing temperature. The reverse occurs with the sesquihydrate [10].

Table VI

Solubility Data of Colchicine at Room Temperature

<u>Solvent</u>	<u>Approximate Solubility (g/ml)</u>
water	1 g/22 ml [5] 1 g/20 ml [7,10] 1 g/25 ml [6]
ether	1 g/220 ml [5,6] 1 g/160 ml (15.5°) [7,9,10]
benzene	1 g/100 ml [5]
alcohol	freely soluble [5,6,7,9,10]
chloroform	freely soluble [5,6,7,9,10]
petroleum ether	practically insoluble [5,10]

3.7 Moisture Content

The Karl Fischer titration procedure is the official United States Pharmacopeia XX [2] method for the drug substance.

Loss on drying at 105°C for 3 hours has been reported [4]. Colchicine has also been dried over phosphorus pentoxide at a pressure not exceeding 0.7 kPa (about 5 torr) for 24 hours [9].

3.8 Specific Rotation

The specific rotation measured at the sodium D line (approximately 589 nm), determined in an aqueous solution containing 100 mg in each 10 ml at 25°C, is between -435° and -460° calculated on an anhydrous, solvent-free basis according to the United States Pharmacopeia XX [2]. The specific rotation given in the British Pharmacopoeia [9] for a 1% aqueous solution at 19.5°-20.5°C is -425° to -450°. It has been reported as -410° to -435° at 25°C [6]. Specific rotation in chloroform is determined as -121° (C = 0.9 m) at 17°C [5]; -119.9° (C = 0.878) at 13°C [1].

3.9 pKa

The pKa at 20° is 12.35 [5,6]. $K = 4.5 \times 10^{-13}$; pH of a 5% solution = 5.9 [5].

3.10 Crystal Properties

The crystal structure [11], conformation, bond distances, bond angles, and atomic coordinates of colchicine are presented in Figures 7, 8, 9, 10, and Table VII, respectively. Crystals grown by slow evaporation of an aqueous solution containing tris(hydroxymethyl)aminomethane (tris) buffer were found to be the dihydrate $C_{22}H_{25}NO_6 \cdot 2H_2O$, monoclinic, space group $P2_1$, $a = 17.08 \pm 0.01$, $b = 10.700 \pm 0.007$, $c = 13.88 \pm 0.01 \text{ \AA}$, $\beta = 117.9 \pm 0.1^\circ$. Unit-cell parameters and standard deviations were obtained from least squares analysis of diffractometer angle measurements. The measured density ($D_m = 1.32 \text{ g/cm}^3$) indicated $Z = 4$ (calculated density $D_x = 1.29 \text{ g/cm}^3$); therefore, the asymmetric unit consists of two independent colchicine and four water molecules. Intensity data were collected from a crystal of $0.3 \times 0.3 \times 0.1 \text{ mm}$. A Syntex $P2_1$ diffractometer was used with monochromated Cu $K\alpha$ ($\lambda = 1.54178 \text{ \AA}$) x-rays.

4. Isolation

Natural product: Colchicine is the medicinally active component in Colchicum autumnale L (Liliaceae) as well as more than fifty species of the Liliaceae family (Melanthoideae subfamily, tribe: Colchiceae).

Alcoholic Extraction

The corm or seed is extracted with alcohol. After distilling off the alcohol, the syrupy residue is diluted

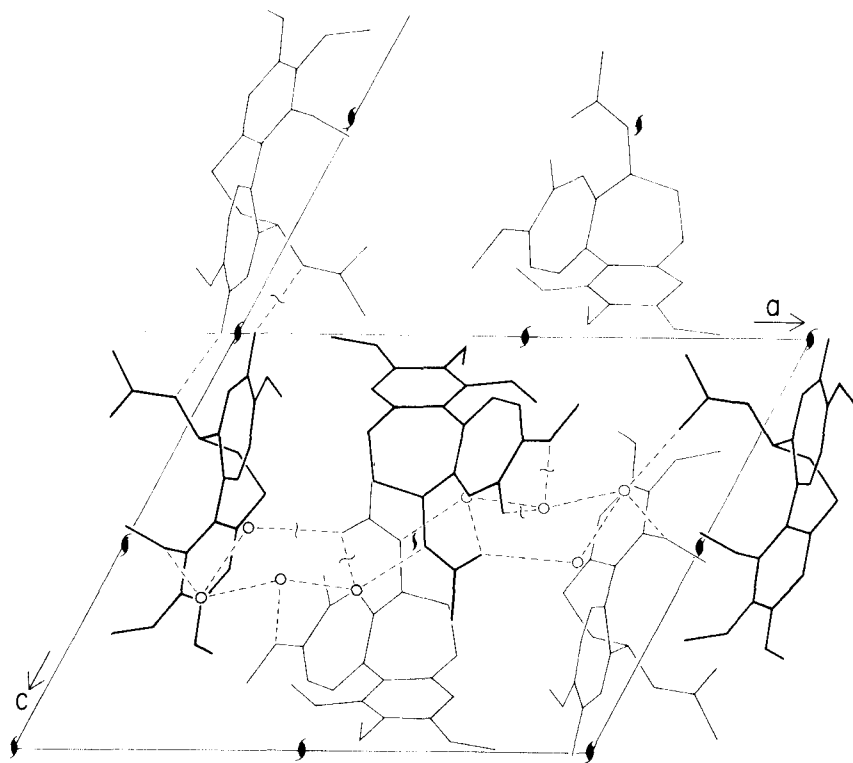


Fig. 7. Projection of the crystal structure down b . Circles are water molecules. Hydrogen atoms are omitted for clarity. Lightly inked molecules are related to heavily inked ones by a twofold screw operation. Dashed lines indicate hydrogen bonds. Symbol \sim indicates hydrogen bonds to molecules related to molecules shown by translation up or down in y . $O(W4)$, $O(1)(a)$, and $O(2)(a)$ are connected by a single bifurcated hydrogen bond.

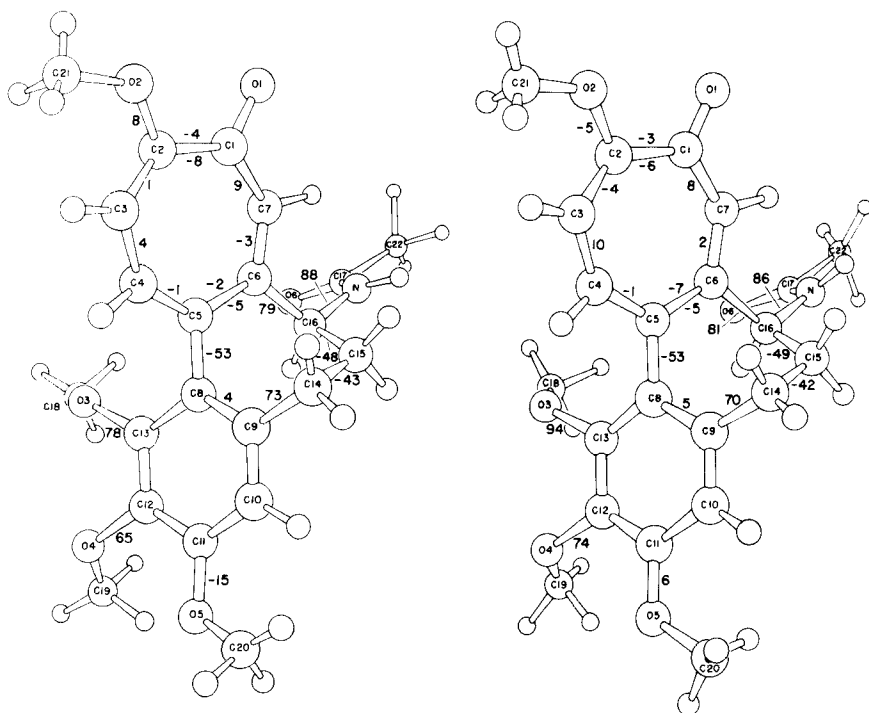


Fig. 8. Experimentally determined conformations of molecule a (left) and molecule b (right), viewed normal to the planes defined by C(5), C(8), C(15). Torsion angles ($^{\circ}$) are given for rings B and C, for O(1)-C(1)-C(2), for C(6)-C(16)-N-C(17), and for the methoxy groups. The latter are taken to be the angles between the planes COCH₃ and the least-squares planes for rings A and C.

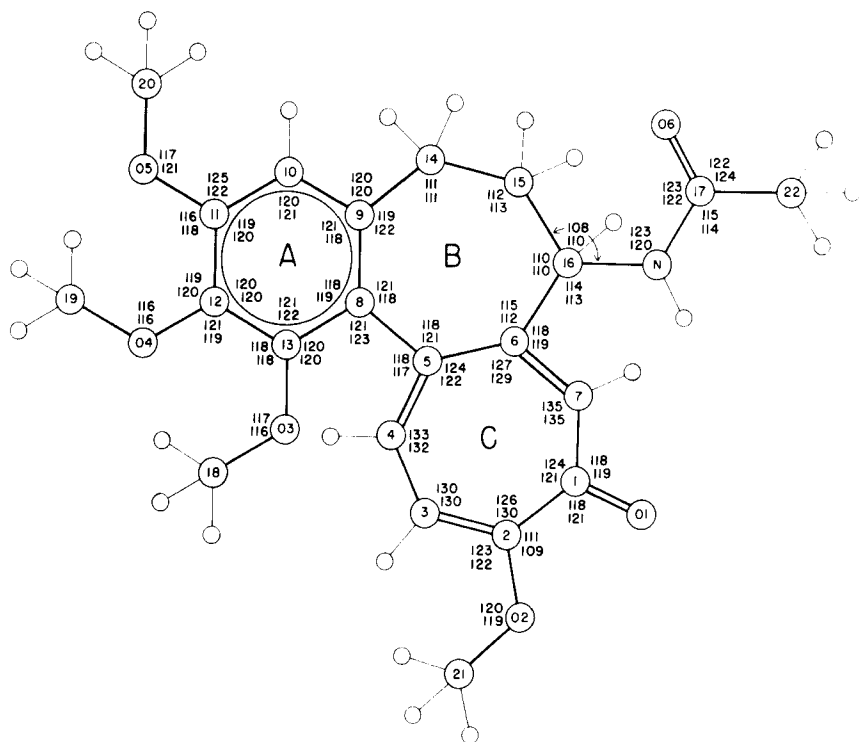


Table VII. Atomic coordinates ($\times 10^4$)

Standard deviations estimated from the least-squares calculations, assuming random errors in the intensity data, are given in parentheses as deviations in the last significant figure.

Molecule <u>a</u>	<u>x</u>	<u>y</u>	<u>z</u>	Molecule <u>b</u>	<u>x</u>	<u>y</u>	<u>z</u>
C(1)	5867 (6)	8131	3535 (7)	C(1)	528 (5)	5109 (8)	988 (7)
C(2)	5910 (6)	8237 (10)	2512 (7)	C(2)	903 (5)	6198 (8)	1674 (7)
C(3)	5520 (6)	7449 (9)	1637 (6)	C(3)	1230 (5)	6316 (8)	2747 (6)
C(4)	5004 (6)	6367 (9)	1488 (6)	C(4)	1362 (5)	5422 (8)	3543 (6)
C(5)	4768 (5)	5714 (9)	2165 (6)	C(5)	1099 (5)	4187 (7)	3477 (6)
C(6)	5027 (5)	6080 (10)	3296 (7)	C(6)	598 (5)	3556 (7)	2435 (6)
C(7)	5495 (6)	7089 (11)	3823 (7)	C(7)	387 (5)	3965 (8)	1436 (6)
C(8)	4191 (6)	4623 (9)	1727 (6)	C(8)	1345 (5)	3519 (8)	4520 (6)
C(9)	3401 (6)	4547 (10)	1775 (6)	C(9)	1794 (5)	2368 (8)	4693 (6)
C(10)	2830 (6)	3540 (12)	1317 (7)	C(10)	2052 (5)	1749 (8)	5686 (7)
C(11)	3048 (6)	2582 (11)	815 (7)	C(11)	1878 (5)	2277 (9)	6492 (6)
C(12)	3869 (6)	2621 (10)	802 (7)	C(12)	1423 (5)	3376 (8)	6291 (6)
C(13)	4413 (6)	3636 (10)	1222 (6)	C(13)	1160 (5)	3984 (8)	5321 (6)
C(14)	3166 (5)	5556 (10)	2337 (7)	C(14)	1963 (5)	1778 (8)	3835 (6)
C(15)	3728 (6)	5478 (10)	3563 (7)	C(15)	1092 (6)	1299 (8)	2882 (7)
C(16)	4718 (5)	5213 (9)	3903 (6)	C(16)	313 (5)	2228 (8)	2570 (6)
C(17)	5947 (6)	4648 (12)	5658 (8)	C(17)	-1276 (6)	2066 (10)	1448 (7)
C(18)	5890 (6)	2991 (10)	1792 (9)	C(18)	-200 (6)	5077 (12)	4647 (10)
C(19)	4210 (6)	467 (10)	748 (8)	C(19)	580 (8)	3362 (12)	7256 (9)
C(20)	1623 (7)	1627 (13)	85 (10)	C(20)	2526 (6)	491 (9)	7704 (7)
C(21)	6581 (6)	9462 (10)	1623 (7)	C(21)	1342 (7)	8328 (9)	1543 (8)
C(22)	6359 (6)	4831 (12)	6872 (7)	C(22)	-2012 (6)	1488 (11)	455 (7)

Table VII -- Cont'd.

Molecule <u>a</u>				Molecule <u>b</u>			
	<u>x</u>	<u>y</u>	<u>z</u>		<u>x</u>	<u>y</u>	<u>z</u>
N	5216 (5)	5309 (9)	5072 (6)	N	-471 (5)	1787 (6)	1590 (5)
O(1)	6228 (4)	8991 (7)	4236 (5)	O(1)	304 (4)	5149 (6)	-6 (5)
O(2)	6395 (4)	9247 (6)	2524 (4)	O(2)	898 (3)	7179 (6)	1033 (4)
O(3)	5165 (4)	3706 (6)	1089 (4)	O(3)	736 (4)	5119 (5)	5177 (4)
O(4)	4073 (4)	1683 (6)	263 (4)	O(4)	1255 (4)	3920 (6)	7095 (4)
O(5)	2507 (4)	1555 (8)	300 (5)	O(5)	2126 (4)	1708 (6)	7479 (4)
O(6)	6286 (4)	3998 (8)	5254 (5)	O(6)	-1398 (4)	2783 (7)	2067 (5)
O(<u>W1</u>)	8016 (4)	3645 (6)	5401 (5)	O(<u>W3</u>)	5459 (5)	1839 (9)	3814 (5)
O(<u>W2</u>)	8179 (5)	2203 (7)	3685 (5)	O(<u>W4</u>)	6893 (9)	1142 (11)	4086 (9)

with water to precipitate the insoluble fats and resins, and filtered. The aqueous solution is then repeatedly extracted with chloroform [1] or digested with lead carbonate, refiltered, evaporated to a small volume, and extracted with chloroform [6]. The colchicine is recovered as a crystalline addition complex with chloroform. The chloroform is then distilled off in steam or alcohol. Amorphous colchicine is produced upon evaporation of the residual solution. Amorphous colchicine may be crystallized from ethyl acetate as pale yellow needles.

Modifications

1. Chromatographic purification of the chloroform solution on alumina [1].
2. Extraction of the dried powder derived from saffron plant parts with petroleum ether to remove fats followed by alcoholic extraction [1].
3. Wax and paraffin wax for the removal of resin [42].
4. Soxhlet apparatus [42].

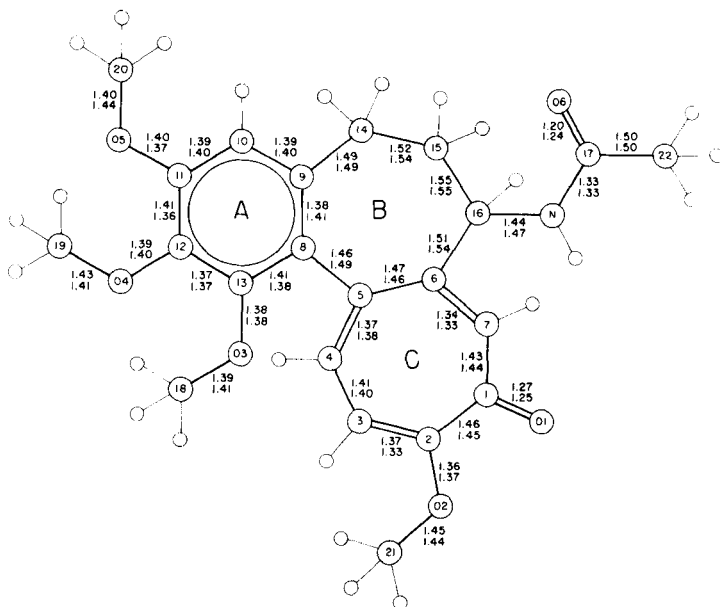
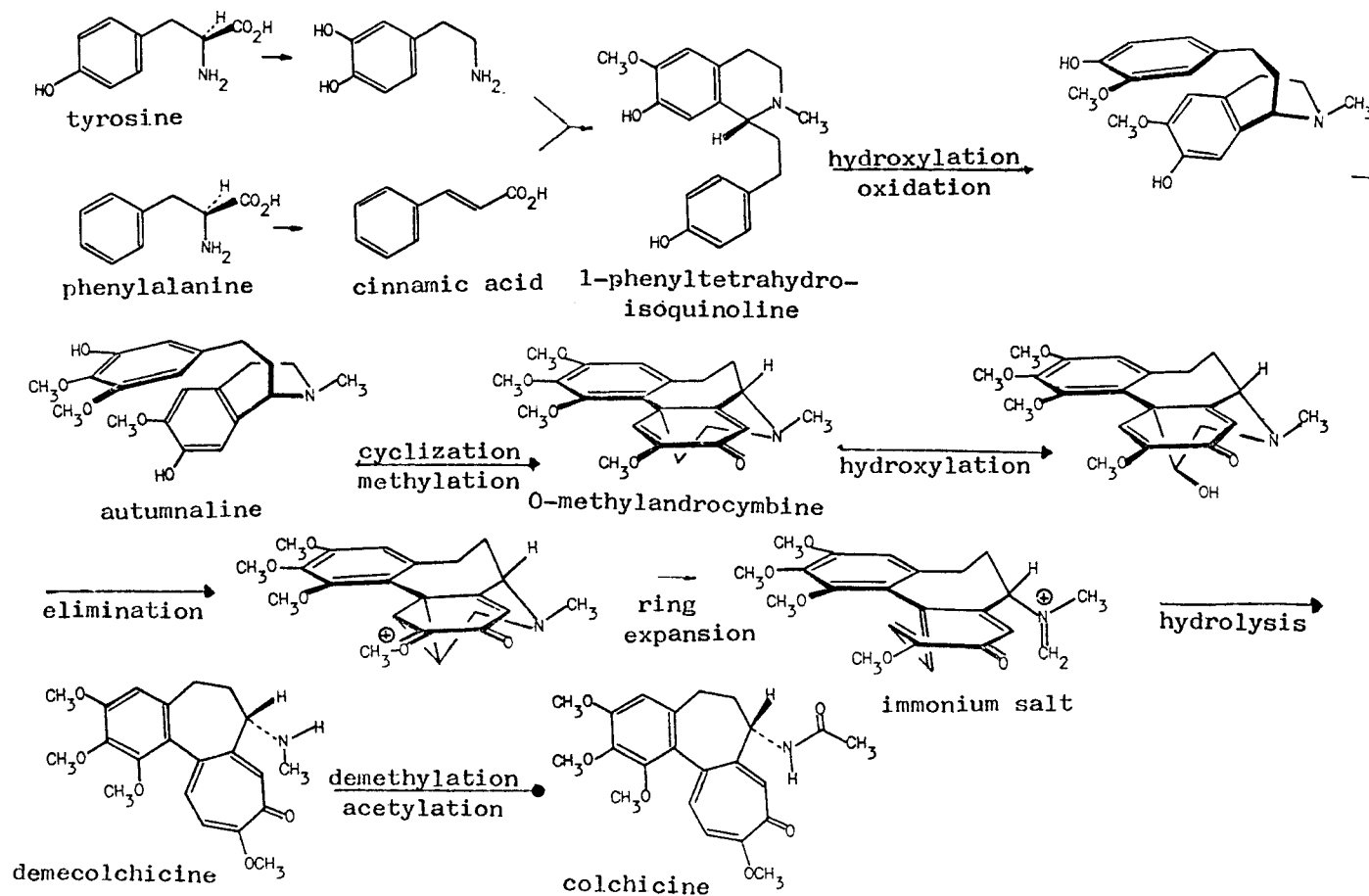


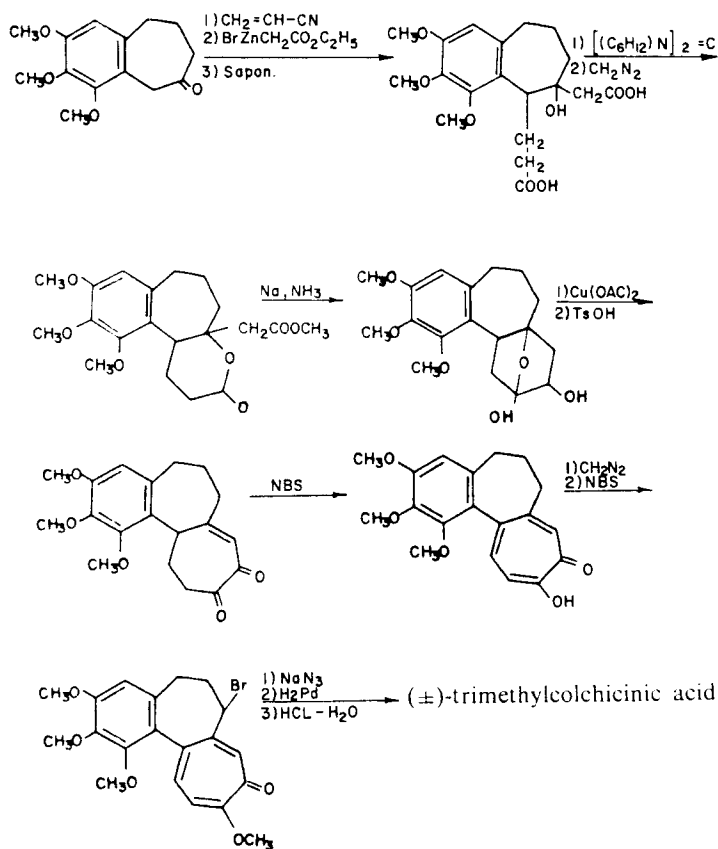
Fig. 10. Bond angles ($^{\circ}$); those for molecule a are listed above those for molecule b. Standard deviations are approximately 1° for each angle. Numbered atoms without element symbol are carbon, unlabelled atoms are hydrogen.

5. Biosynthesis of Colchicine [22,23]



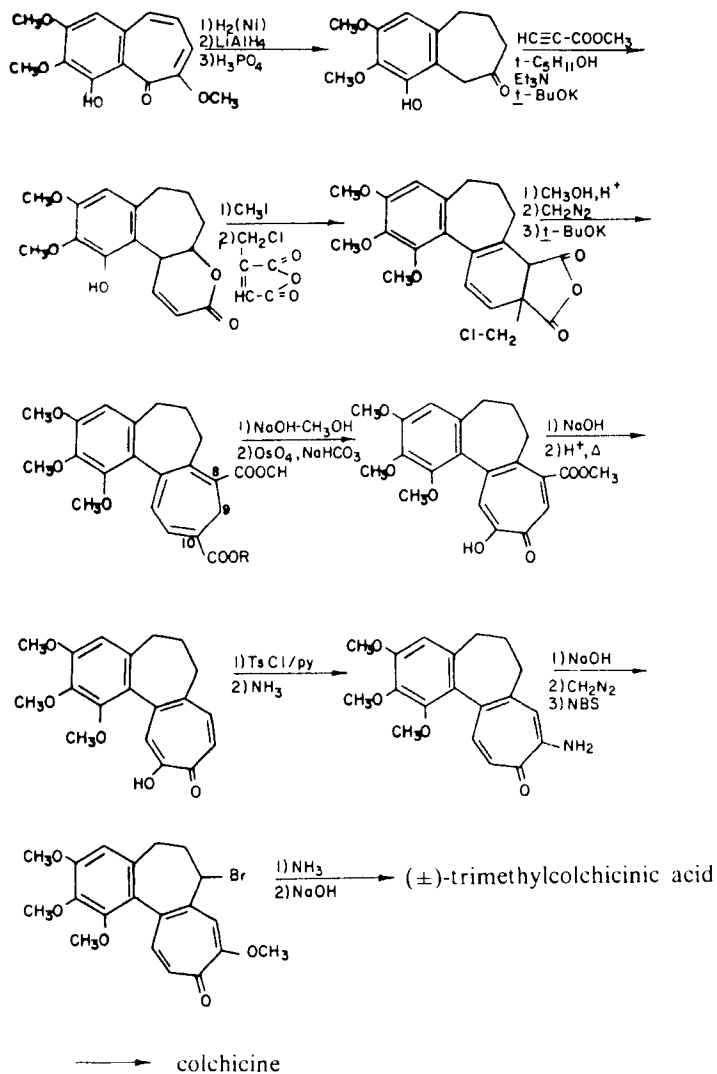
6. Synthesis

6.1 Synthesis from 7,8,9-Trimethoxy-9-benzosuberone [22].

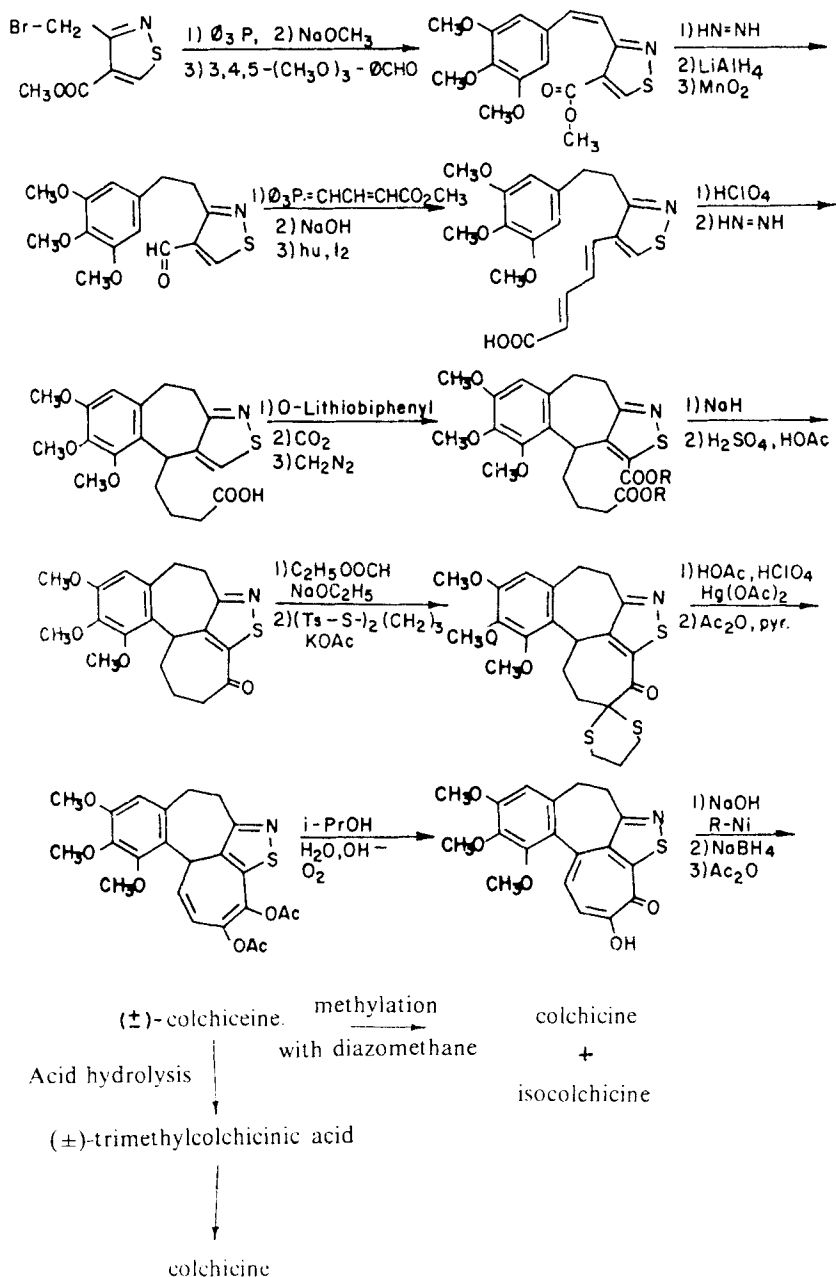


resolution, N-acetylation, and O-methylation. \longrightarrow

6.2 Synthesis from Purpurogallin trimethyl ether [22].



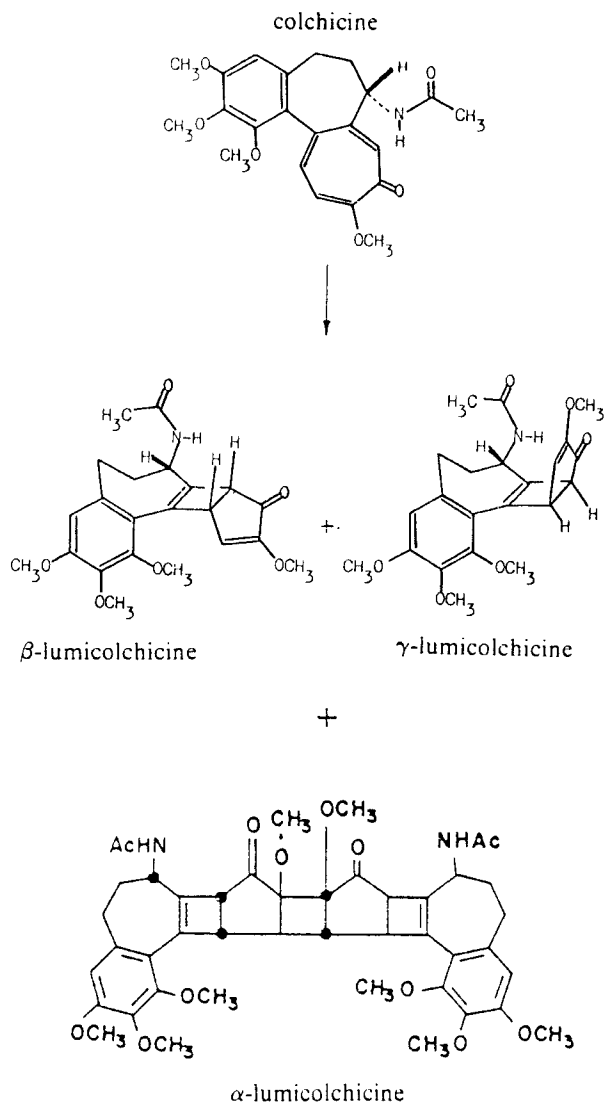
6.3 Synthesis from a Substituted Isothiazole [22].

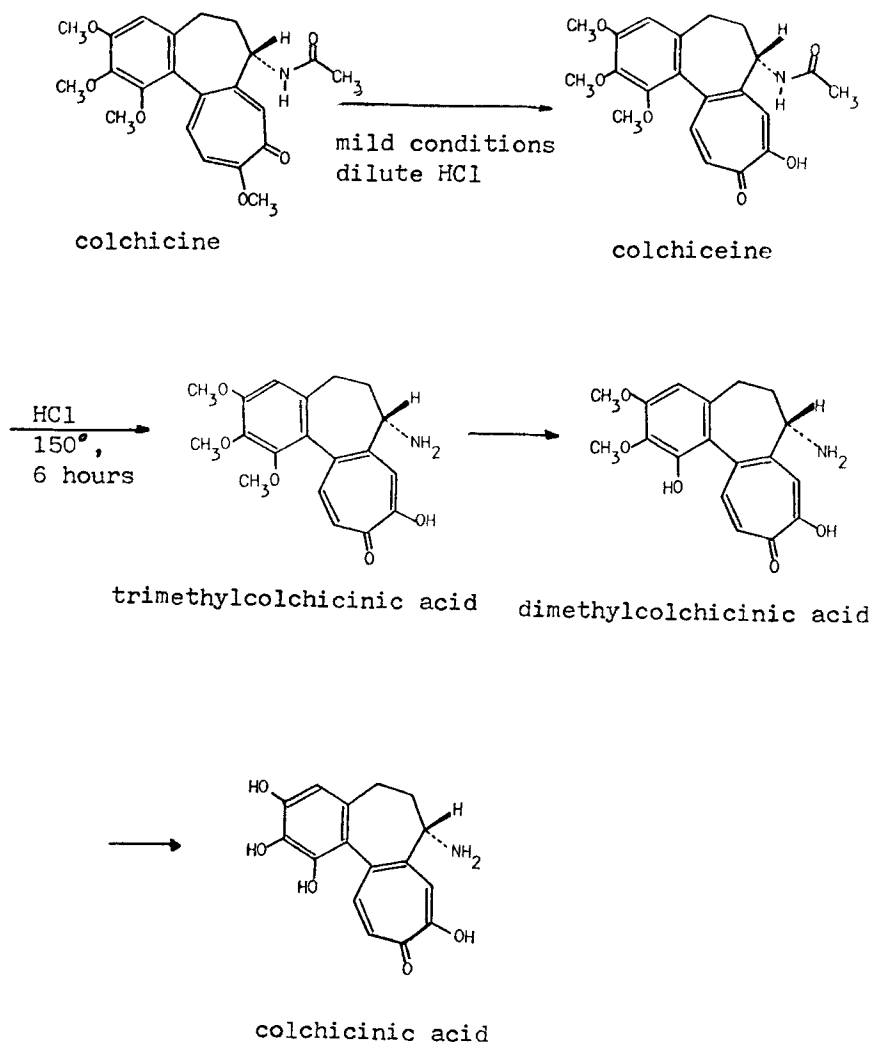


7. Stability and Degradation

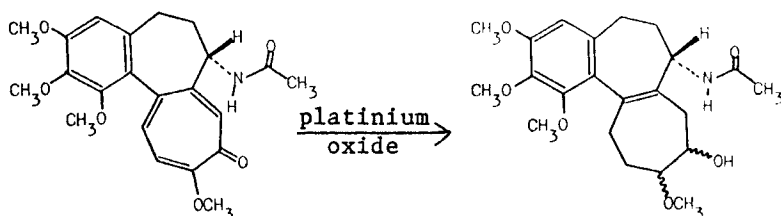
7.1 Reaction with Ultraviolet Light [22,23]

Colchicine is converted into a mixture of three photoisomers in the presence of ultraviolet light. A tetracyclic structure is formed with loss of the tropanoid ring.



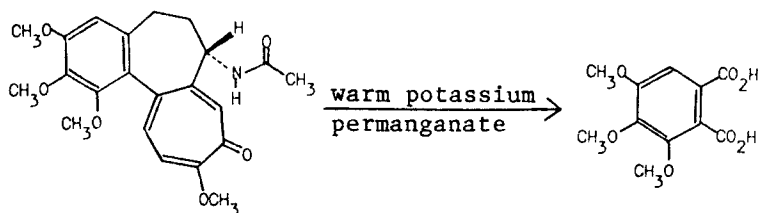
7.2 Acid Hydrolysis [23,1]

NOTE: Conversion of colchicine to colchicineine and other products also occurs during alkaline hydrolysis pH >13 [24], there is no appreciable hydrolysis to colchicine occurring in neutral or slightly alkaline (pH 8.1) solutions even after 2 months storage [9].

7.3 Hydrogenation [23,1]

colchicine

hexahydrocolchicine

7.4 Oxidation (23)

colchicine

3,4,5-trimethoxyphthalic acid

8. Metabolism

Little is known regarding colchicine absorption from the gastrointestinal tract [7]. A number of studies do indicate that a metabolite is the active form of the drug. A study of metabolism in gout patients suggests that a major portion of the drug is deacetylated but it is not known whether deacetylcolchicine is the human metabolite [18]. Colchicine and desmethycolchicine have been found in the bile [39].

There is no evidence of specific accumulation in certain tissues [7]. However, colchicine's ability to disrupt mitosis is well known and post-mortem examination indicates arrested metaphases in the lymph glands, spleen,

and liver, as well as in bone marrow and in duodenal mucosa [1]. After two hours, the highest concentrations of colchicine have been found in the liver and kidney in the dog, rat, rabbit, and hamster; the brain had the lowest concentration [39]. The ability to arrest mitosis is believed to result from strong binding of the drug to the protein tubulin which prevents the assembly of the tubulin into microtubules which form the mitotic spindle [11].

Although metabolites are not well known, it has been postulated that colchicine inhibits the acute gouty attack by inhibiting phagocytosis of urate crystals by leukocytes, thus diminishing the inflammatory reaction [35].

Colchicine also induces polyploidy in plants and malformations of embryos [1,11].

9. Pharmacokinetics

Humans excrete 5-50% of the injected dose within 48 hours [39]. About 50% of the injected dose is recovered from the mouse within 16 hours. The bulk of the colchicine is excreted within the first 24 hours, especially at high blood levels. Colchicine may be excreted in the bile or directly into the gastrointestinal tract [7,39], although bile seems to be an important pathway for the excretion of colchicine [39]. In mice, 2.4-14% of the dose is excreted in the feces [7]. Higher amounts are excreted in the feces of rats.

Dose -- 1 mg initially; subsequent doses of 500 μ g every two hours until pain is relieved or until toxic effects such as vomiting or diarrhea are experienced; IP max in 24 hours is 3 mg.

Colchicine frequently causes nausea, vomiting, and diarrhea. Larger doses may cause dehydration and hypotension. Hair loss may result after prolonged treatment. Symptoms of poisoning may be observed after 3-6 hours [9,34]. Fatality has resulted from ingestion of as little as 7 mg [34]. Death generally results in 7 to 36 hours [9].

10. Methods of Analysis10.1 Elemental Analysis [64]

	<u>theory</u>	<u>found</u>
carbon	66.15	65.40
hydrogen	6.31	6.30
nitrogen	3.51	3.35
oxygen	24.03	23.97

10.2 Color Tests

<u>Agent</u>	<u>Color</u>	<u>Reference</u>
1 dilute mineral acids and alkalis	intense yellow	1
2 nitric acid	violet slowly changing to yellow then to green	1
3 sulfuric acid-formaldehyde	yellow (sensitivity 0.25 μ g)	7
4 ammonium molybdate	yellow (sensitivity 0.25 μ g)	7
5 ammonium vanadate (Vitali's test)	green (sensitivity 0.25 μ g) yellow \rightarrow purple/brown/red-brown (sensitivity 0.25 μ g)	7 7
6 ferric chloride T.S.-alcohol	garnet red	4,9
7 sulfuric acid followed by nitric acid	lemon-yellow greenish-blue \rightarrow reddish \rightarrow yellow or almost colorless	9
8 excess of sodium hydroxide	red	4,9
9 water (color intensified by adding mineral acids)	yellow	9

	<u>Agent</u>	<u>Color</u>	<u>Reference</u>
10	nitric acid-water-sodium hydroxide	orange red	34
11	concentrated nitric acid; addition of water; followed by sodium hydroxide	violet → brown/red → yellow → orange/red	42
12	hydroxylamine-sodium hydroxide (warm the solution)	orange	43

10.3 Aqueous Titrimetric Analysis (Residual Titration)

An accurately weighed sample of colchicine is dissolved in excess 0.02 N hydrochloric acid. The excess acid is titrated with 0.02 N sodium hydroxide using methyl orange as indicator [47].

10.4 Non-aqueous Titrimetric Analysis

The non-aqueous titration procedure is the official United States Pharmacopeia XX [2,9] and the British Pharmacopoeia method for the drug substance. An accurately weighed sample of colchicine is dissolved in a mixture of acetic anhydride-toluene (1:2). The end-point is determined potentiometrically using 0.02 N perchloric acid as the titrant. An additional non-aqueous titration procedure was presented [47] in which glacial acetic acid containing 3-4 drops of acetic anhydride is used to dissolve colchicine. Titration is accomplished using either crystal violet or potentiometric determination using calomel and glass electrodes; 0.01 N perchloric acid is used as the titrant.

10.5 Spectrophotometric Analysis

The official USP XX [2] method for the analysis of Colchicine Tablets is spectrophotometric. A portion of powdered tablets is weighed and colchicine is extracted with chloroform from an aqueous solution. The UV spectrum of the chloroform solution is recorded and compared to the USP reference standard (diluted to the same final concentration with chloroform) at the maximum absorbance at about 350 nm. Spectrophotometric analysis also was conducted using nitric acid to dissolve the drug, followed by sodium hydroxide T.S. and dilution with water; the solution was

read at 350 nm, with an additional maximum observed at about 510 nm [42].

Analysis using the hydroxylamine-sodium hydroxide color reaction (orange color) was accomplished using readings taken at 500 nm wavelength [43]. Ferric chloride solutions, after acid hydrolysis, were read at 470 nm [48, 49]. Colchicine was also analyzed after lithium aluminum hydride reduction and extraction from 1% hydrochloric acid-ammonia-acetic acid solution into carbon disulfide. The organic layer was removed and combined with benzene. Readings are at 445 nm [47].

Isonicotinic hydrazide in alkaline media has also been used for reaction with colchicine for a colorimetric determination [34].

10.6 Fluorescence Analysis

The emission and excitation spectra are provided in Figure 11. The emission maximum is 422 nm. It was obtained using a Perkin Elmer MPF-2A spectrofluorometer with a slit at 6 nm [50] and the excitation wavelength at 350 nm [67]. The emission maximum is shifted towards the red as the solvent polarity is decreased [67].

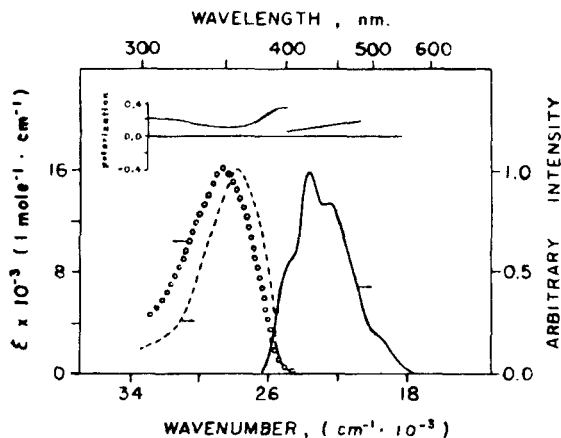


Fig. 11. Absorption (000, 296 K), excitation (---, 77K) and emission (----, 77K) spectra for colchicine in EPA. The excitation is not corrected. The inset shows the polarization of the excitation and emission spectra.

Table VIII

Thin-layer Chromatography of Colchicine

<u>Plate</u>	<u>Solvent</u>	<u>Method of Detection</u>	<u>Rf x 100</u>	<u>Ref.</u>
silica gel F-254	chloroform-acetone- diethylamine (5:4:1)	A,B,C,D,E	59 47	8 54,55
silica gel F-254	chloroform-methanol- acetic acid (85:15:1)	A,B,C,D,E	75	8
silica gel F-254	chloroform-methanol (9:1)	A,B,C,D,E	64 68	8 39
aluminum oxide F-254	chloroform-acetone- aqueous ammonia (25:20:0.4)	A,B,C,D,E	64	2,8
silica gel F-254	chloroform-methanol- diethylamine (5:4:1)	A,B,C,D	98	8
silica gel F-254	toluene-ethanol- aqueous* ammonia (170:28:2)	A,B,C,D,F	18	8
silica gel G	benzene-acetone- ether-10% aqueous ammonia (4:6:1:0.3)	-----	15	54

Table VIII -- Cont'd.

<u>Plate</u>	<u>Solvent</u>	<u>Method of Detection</u>	<u>Rf x 100</u>	<u>Ref.</u>
silica gel G	benzene-acetone- ether-25% aqueous ammonia (4:6:1:0.3)	-----	20	54
silica gel G	chloroform-diethyl- amine (9:1)	G	41	54,55
alumina G	chloroform	G	11	54,55
silica gel G pre- treated with 0.1 N sodium hydroxide	methanol	G	57	54,55
silica gel G	benzene-ethyl acetate- diethylamine (5:4:1) + 8% methanol	B,G,H	56 46	56,5 39
	chloroform-acetone- diethylamine (7:2:1)	B,G,H	61 60	56,5 39
silica gel G	methanol-aqueous ammonia (100:1.5)	I	62	7

10.7 Polarographic Analysis

A polarographic analysis of colchicine was accomplished using a dropping mercury working electrode and a silver wire reference electrode in 0.1 M tetrabutylammonium iodide [53,52]. The dc and ac polarographic responses are recorded. Half wave potential is -1.47 volts. Detection limits for dc: 3×10^{-4} moles per liter; ac: 1×10^{-5} moles per liter; ac second harmonic: 5×10^{-7} moles per liter.

10.8 Thin-Layer Chromatography

Thin-layer chromatography has frequently been used for the analysis of colchicine. Methods of detection and solvent systems are listed in Table VIII.

10.9 Paper Chromatography

Ascending paper chromatography was accomplished using Whatman #1 paper which was dipped in a 5% solution of sodium dihydrogen citrate and dried. The solvent consisted of 4.8 g of citric acid in a mixture of 130 ml of water and 870 ml of 1-butanol ($R_f \times 100 = 83$). Examination was conducted using shortwave ultraviolet light [7]. An additional analysis was obtained using formamide/benzene-chloroform-formamide (7:3:1) and longwave ultraviolet detection [57].

10.10 Gas Chromatography

The following GLC systems have been used for analysis; however, thermal instability of colchicine has been observed.

<u>Detector</u>	<u>Phase and Column</u>	<u>Temperature (°C)</u>	<u>Ref.</u>
FID	5% SE-30 on 60-80 mesh Chromosorb WAW	230	7,54
	5-ft x 1/8-inch stainless steel col. (31 ml/min nitrogen)	250, 270	54
HFI	1% Hi-Eff-8B on 100/120 mesh silanized Gas Chrom P 0.9-m x 3.2-mm glass columns (approximately 60 ml/min nitrogen)	220, 250	54

Methods of Detection

- A. shortwave ultraviolet light
- B. longwave ultraviolet light
- C. 0.5% iodine in chloroform
- D. 40% sulfuric acid in methanol followed by heat (105°)
- E. 40% sulfuric acid in methanol followed by heat (105° C)
and longwave ultraviolet light
- F. acidified potassium iodoplatinate
- G. potassium iodoplatinate
- H. antimony (III) chloride
- I. p-dimethylaminobenzaldehyde

10.11 High-Performance Liquid Chromatographic Analysis

High-performance liquid chromatography has been used extensively for the analysis of colchicine. The various HPLC systems used for the analysis are given in Table IX.

10.12 Related Alkaloids

The related alkaloids procedure is the official USP XX method [2] for the drug substance. Two solutions of differing concentrations of colchicine in alcohol are compared by thin-layer chromatography using an alumina plate with a fluorescent indicator (254 nm) and a mobile solvent consisting of chloroform-acetone-aqueous ammonia (25:20:0.4). Comparison of the two samples is accomplished using shortwave ultraviolet light.

10.13 Determination of Occluded Solvents (chloroform and ethyl acetate)

The occluded solvents procedure is the official USP XX limit test for the drug substance [2]. A gas chromatographic procedure using a flame-ionization detector, 20% polyethylene glycol (950-1050 average molecular weight) on flux-calcined diatomaceous earth, and nitrogen carrier gas is used. The column is maintained at 75°. An aqueous colchicine solution with added internal standard (n-propyl alcohol) is compared to known quantities of aqueous ethyl acetate-chloroform solution (n-propyl alcohol added).

11. Determination in Biological Fluids

11.1 Bile

Colchicine has been determined in bile by thin-layer chromatography on silica gel F-254 using ultraviolet detection and the systems listed below [39]. Colchicine was separated from colchicine, desacetylcolchicine, and desmethycolchicine.

Systems

methanol-chloroform (9:1) $R_f = 0.68$
benzene-ethyl acetate-diethylamine (5:4:1) and 8%
methanol $R_f = 0.46$
chloroform-acetone-diethylamine (7:2:1) $R_f = 0.60$.

Table IX

High Performance Liquid Chromatography Systems for Colchicine

<u>Column</u>	<u>Mobile Phase</u>	<u>Flow/ Temperature</u>	<u>UV Detection Pressure</u>	<u>Wavelength</u>	<u>Ref.</u>
Zorbax-Sil	87-89% methylene chloride-2-propanol	ambient	42 atm	254	8
Partisil 10/25	85-90% methylene chloride-2-propanol	ambient	1000 psi	254	8
Chromanetics C8	methanol-water (1:3)	ambient	1000 psi	254	8
LiChrosorb RP-8	30% acetonitrile-water	ambient	925-1550 psi	254	8
Partisil ODS	methanol-water (1:1,1:2)	ambient	1000 psi	254	8
μBondapak C18	20% and 35% acetonitrile-water	ambient	925-1550 psi	254	8
μBondapak C18	acetonitrile-methanol -phosphate buffer pH 7.6 (17:5:78) -phosphate buffer pH 6.0 (17:5:78) -phosphate buffer pH 6.0 (16:5:79) -phosphate buffer pH 6.0 (15:5:80)	ambient	1390 psi (2 ml/min)	350	58

Table IX -- Cont'd.

<u>Column</u>	<u>Mobile Phase</u>	<u>Temperature</u>	<u>Flow/ Pressure</u>	<u>UV Detection Wavelength</u>	<u>Ref.</u>
LiChrosorb Si-60	gradient: aceto- nitrile - 10% aceto- nitrile in water (0-30%)	ambient	2 ml/min	254	59
Octadecylsilane reversed phase column (Waters)	5 mM pentanesulfonic acid in methanol- acetonitrile-phosphate buffer (0.1 M pH 7.6), (41:15:44). The mobile phase was adjusted to a final pH of 6.45 by addition of glacial acetic acid	40°	1.5 ml/min	254	62
Hypersil (5 µm)	dichloromethane- 2-propanol	ambient	2 ml/min	240	63
LiChrosorb RP-18	acetonitrile-methanol- phosphate buffer pH 6.0 (16:5:79)	ambient	3.0 ml/min	350	60

11.2 Urine

Colorimetric analysis after acid hydrolysis followed by addition of ferric chloride reagent has been used. Readings are at 630 nm to minimize interference by colored urine components [49]. Paper chromatography using benzene-formaldehyde on Whatman #1 or #4 paper and concentrated hydrochloric acid spray for detection (yellow green band) after extraction has also been used [49].

11.3 Plasma

HPLC at 240 nm using a Hypersil column and dichloromethane/isopropanol solvent was reported. The plasma was extracted from an aqueous ammonia solution into dichloromethane. Ethanol was added to the dichloromethane after removal of the aqueous layer. The solution was evaporated to dryness and dichloromethane added to dissolve the residue [63].

11.4 Blood

Analysis by HPLC was conducted using octadecylsilane reverse phase columns eluted with pentanesulfonic acid in methanol-acetonitrile-phosphate buffer, pH 7.6 (4:15:44) adjusted to pH 6.45, and UV detection at 254 nm. The colchicine was extracted from sodium bicarbonate solution into dichloromethane [62].

11.5 Microbial Cultures

HPLC using a LiChrosorb RP-18 column with acetonitrile-methanol-phosphate buffer pH 6.0 (16:5:79) mobile phase and UV detection at 350 nm was used for determination of colchicine and colchicine in microbial cultures after extraction from sodium hydroxide solution into chloroform. The chloroform extract was evaporated to dryness and reconstituted in the mobile phase [60].

11.6 DNA

Spectrophotometric analysis in pH 7, 10, and 12 phosphate buffer and proton NMR in D₂O was used to determine the interaction between colchicine and DNA [37].

11.7 Tubulin-Colchicine Complex

Fluorescence was measured in PMC (sodium phosphate

pH 7.0-magnesium chloride) buffer solutions [67].

12. Determination in Pharmaceuticals

The official USP and BP methods are spectrophotometric [2,9]. HPLC and other methods can readily be adapted for quantitative use. Additional procedures such as HPLC involve analysis of the powdered seeds [59].

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CYANOCOBALAMIN

Joel Kirschbaum

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1. Introduction

1.1 History

In 1855, Thomas Addison¹ of Guy's Hospital, London, described a form of "idiopathic anaemia". About 50 years later clinicians found that deficiencies of dietary factors could lead to some diseases in humans. Casimir Funk, in 1911, called these essential compounds "vitamins" (Latin *vita*, life, + amine) because many contained amine functions.

G.H. Whipple², in 1922, reported that beef liver, beef heart and various other food enhanced hemoglobin formation in patients suffering from pernicious anemia, so-called because it usually lead to death.

G.R. Minot and W.P. Murphy³ reported clinical improvement in patients eating daily a special diet containing 120-240 grams of liver. Minot, Murphy and Whipple received the Nobel prize in physiology and medicine in 1934 for these discoveries.⁴

E.J. Cohn and his associates started fractionating⁵ liver using the responses of untreated human patients, each acting as his own control. Normal gastric juice was also administered orally. The food factor was called "extrinsic factor" and the essential substance in gastric juice was called "intrinsic factor", which was unnecessary if large amounts of the food factor were assimilated. Only slow progress was made to obtain purified material until the growth of *Lactobacillus lactic*

Dorner^{6,7} was used to monitor the purification of the "anti-pernicious anemia" factor. In 1948, this simplified analytical method enabled a group of investigators headed by K. Folkers⁸, at Merck & Company, to announce that they isolated from liver a red, crystalline compound, which, in microgram quantities, produced hematological remissions in patients with Addison's pernicious anemia. They named this new agent vitamin B₁₂. Eight days later, E.L. Smith^{9,10} of Glaxo laboratories, independently reported on the purification of an anti-pernicious anemia factor in liver. A single dose of 3 to 6 μ g produced hematological responses in patients with pernicious anemia.

1.2 Structure, Nomenclature and Molecular Weight

Cyanocobalamin is the United States adopted name¹¹. The preferred chemical name¹² is cobinamide cyanide phosphate 3'-ester with 5,6-dimethyl-1- α -D-ribofuranosylbenzimidazole inner salt.

Other names include vitamin B₁₂, 5,6-dimethylbenzimidazolyl cyanocobamide, and Co ^{β} -[α -(5,6-dimethylbenzimidazolyl)]-Co ^{β} -cyanocobamide. Trade names¹³ include Bevatine-12, Berubigen, Betalin-12 crystalline, β -Twelv-Ora, Depinar, Dodecavite, Dodex, Endoglobin, Hepcovite, Normocytin, Poyamin, Rubramin PC, Sytobex, Vibalt, Vitron-C-Plus, Vi-Twel and Tulag.

Cyanocobalamin ⁵⁷Co (CAS 13115-03-2 and 41559-38-0) and ⁶⁰Co (CAS-13422-53-2) are diagnostic aids (also called Rubratope-57 and Rubratope 60), frequently used for the Schilling test; *cf.* section 5.5, Radioactive Methods of Analysis. The molecular weight is 1355.42 daltons; C₆₃H₈₈CoN₁₄O₁₄P. Vitamin B₁₂ is given the chemical abstracts systematic number 68-19-9.

Figure 1 shows the structure and numbering of cyanocobalamin and its most important analogue, coenzyme B₁₂ [α -adenylcobamide coenzyme, α -5,6-dimethyl benziminazolyl)-cobamide 5'-deoxyadenosylcobalamin and cobinamide coenzyme]. The corrin nucleus is shown darker. Corrinoid is a general term used for B₁₂-group compounds. Bound to one

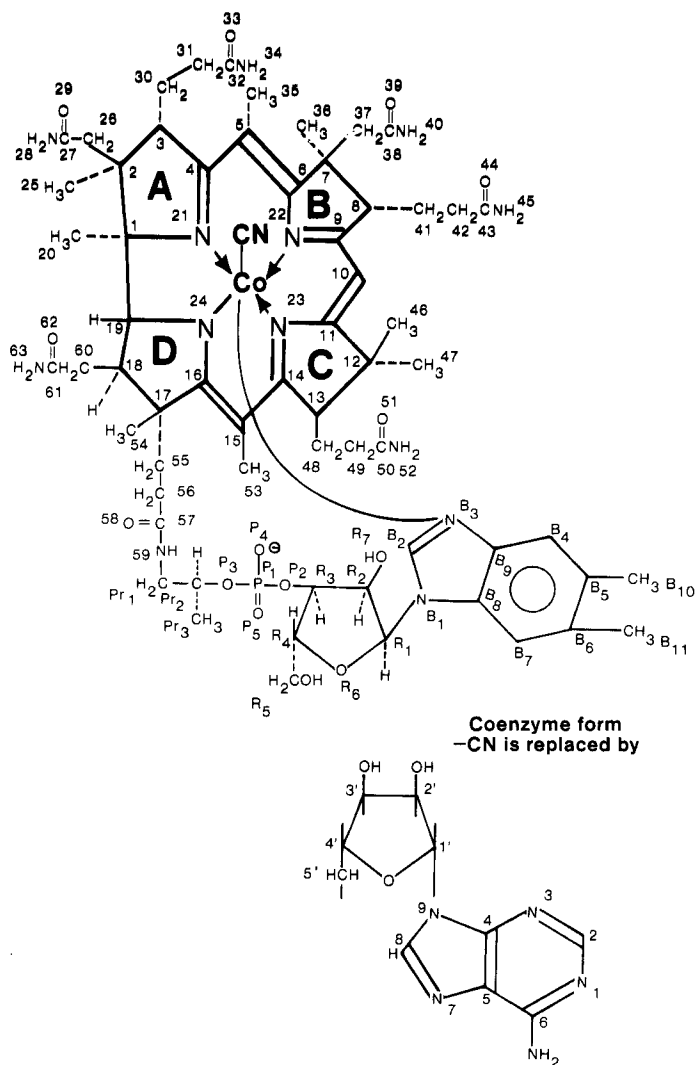
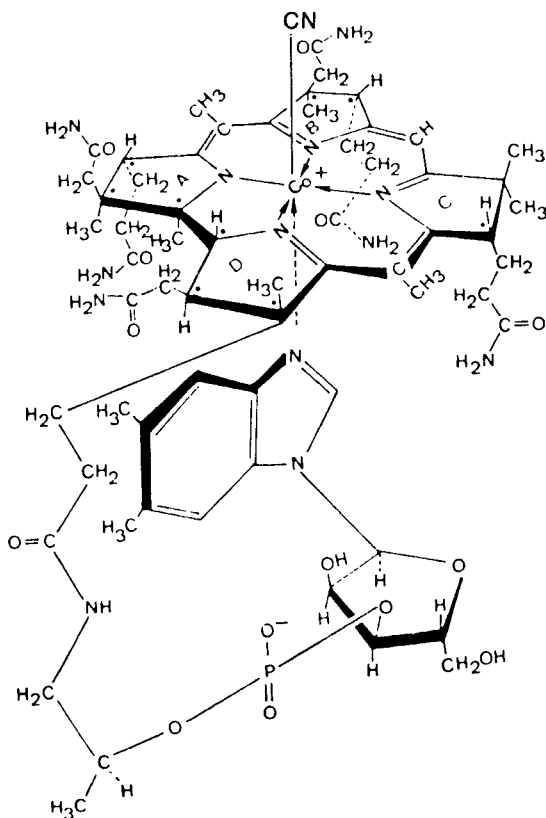


Figure 1. Cyanocobalamin (Vitamin B₁₂)
The corrin nucleus is shown darker.

carboxyl (position f, D ring) is Dg-1-amino-2-propanol (isopropanolamine) which is esterified to the phosphate of 3'-mononucleotide. The sugar is *D*-ribofuranose, which is linked by an α glycoside rather than the usual β -linkage of nucleic acids. The 5,6-dimethylbenzimidazole moiety can be replaced by such organic bases as adenine (B_{12b}), benzimidazole, 5-hydroxybenzimidazole, guanine, 2-methyladenine (B_{12m}), 2-methylhypoxanthine and hypoxanthine, and by OH^- (hydroxocobalamin, B_{12a}), and NO_2^- (nitritocobalamin, B_{12c}); cf. section 16. Reactions of Cyanocobalamin.

Below is another representation of cyanocobalamin, showing the relationship of the corrin nucleus to the other groups, as modified from the frontispiece of reference 18.



1.3 Appearance, Color, Odor and Precautions

Cyanocobalamin is a deep red, odorless, free flowing, crystalline powder. The color has been used to follow purification and to detect the compound. All corrinoids are beautifully colored compounds with colors varying from violet, red and yellow to blue, brown and green.

Since the human dose is approximately 3 micrograms, care should be taken to avoid inhaling the powder, although there is no evidence that cyanocobalamin itself is toxic, even in large doses. Allergic type reactions have been reported only rarely, and it is not known whether those were related to the drug, the excipients, or neither.

1.4 Synthesis, Biosynthesis and Commercial Production

The synthesis of cyanocobalamin was a joint effort by R.B. Woodward^{14,15} and A. Eschenmoser¹⁶. It is shown in abbreviated form in Figure 2. The "cornerstone" of the synthesis was the compound β -corrinorsterone (I) named by Woodward. It was made in a 37 step synthesis and contains 6 chiral centers. The Woodward-Hoffman rules of orbital symmetry for predicting the feasibility, stereochemistry and products of reactions originated with this work. After several other reactions, the resulting cyanobromide (II), containing the A and D rings, was condensed with thiodextrolin (III), which contains the B and C rings, to form IV. Several steps later, the thiolactam IV was freed of sulfur via (V) concomittantly with a mixture of 8 diastereomers. High-pressure liquid chromatography was used at several steps to monitor and purify intermediates in the route to the cyclized, cobalt-containing intermediate VI. Bridgehead structure was introduced stepwise to give VII, which, in several steps including preparative high-pressure liquid chromatography to separate two isomers, was converted to cobyric acid, VIII, containing two cyanide groups.

The side chain was synthesized starting with *D*-ribose (IX), which, in several steps, was converted

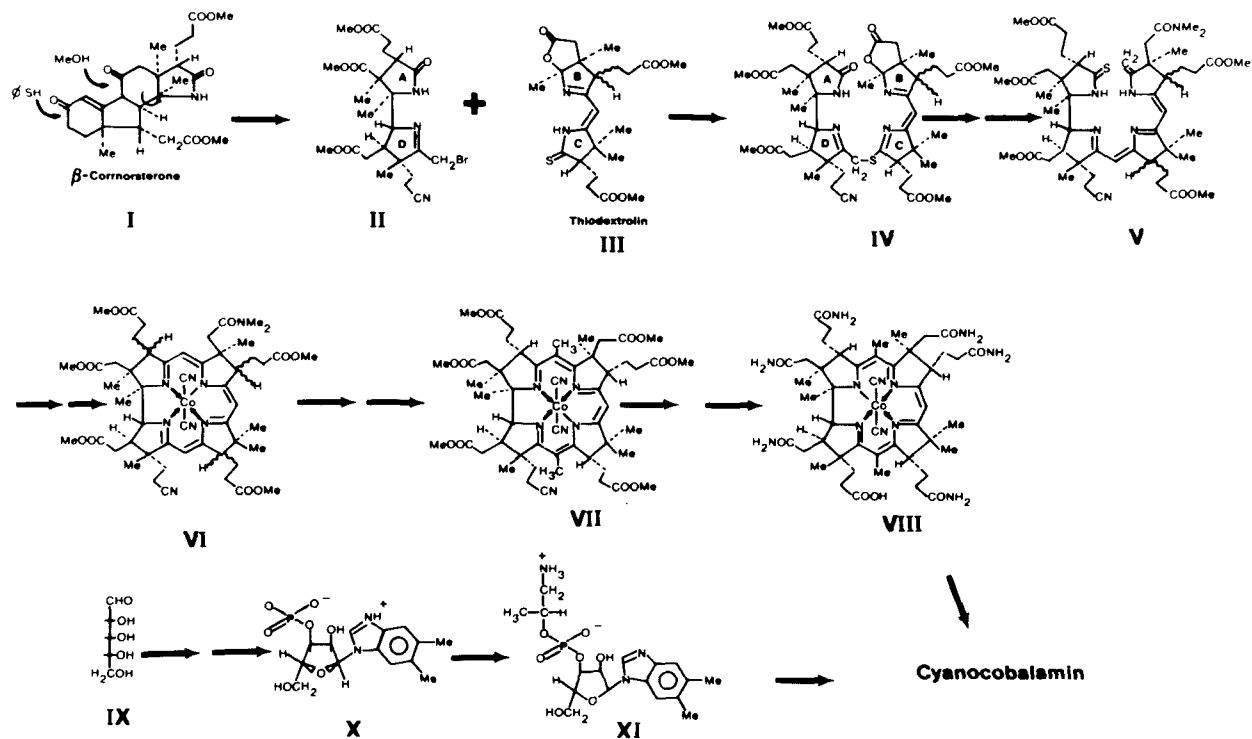


Figure 2. Selected Steps in the Chemical Synthesis of Cyanocobalamin.

to a α -ribazole-3-phosphate (X). Following conversion to the *D*-1-amino-2-propanol ester (XI), condensation with cobyrinic acid(VIII) yields cyanocobalamin. Details of this synthesis have been reviewed.^{17,18}

The biosynthesis of cyanocobalamin begins with δ -aminolevulinic acid (Figure 3, I)¹⁹, which is also the precursor of the porphyrins, chlorophyll, heme and the cytochromes. These pigments are metal complexes involved in oxygen, electron or redox reactions. I was enzymatically converted to porphobilinogen, II. A deaminase condenses 4 moles of II to form the linear molecule bilane (III), where $X = \text{NH}_3^+$. III was converted to uro'gen-III (IV). The order of assembly was investigated using NMR, III, IV, and the deaminase partially saturated with ^{13}C -II. Apparently, the first unit²⁰ of II to bind to the enzyme became ring A, the second unit became ring B, the third C and the final one, D. Following cyclization to IV, ring D rearranged to dihydrosirohydrochlorin, V. (An alternative pathway from IV leads to protoporphyrin IX, and the porphyrins). The methyl groups are from *S*-adenosylmethionine. Further methylation²¹ gave VI, which subsequently lost the methylene group at C-20, as the carboxyl carbon of acetic acid,²² to form cobyrinic acid (VII). Decarboxylation at C-12 also occurred prior to cobyrinic acid formation. Cobyrinic acid, the biological precursor of cyanocobalamin, is closely related to cobyrinic acid (Figure 2, VIII) which was a key intermediate in the chemical synthesis of vitamin B₁₂.

L-Threonine is the precursor of (R)-1-amino-2-propanol, from studies using labeled amino acid. The 5,6-dimethylbenzimidazole moiety and riboflavin appear to have a similar biogenetic source. There also exist pathways in many organisms to convert one corrinoid into another by a net exchange of free and bound base.²³ It has been suggested²⁴ that vitamin B₁₂ is an artifact and the naturally occurring, interconvertible form is the related coenzyme, adenosylcobalamin.

Cyanocobalamin is produced by a large number of diverse microorganisms.^{25,26} The vitamin is usually adsorbed on charcoal, calcium montmorillonite,

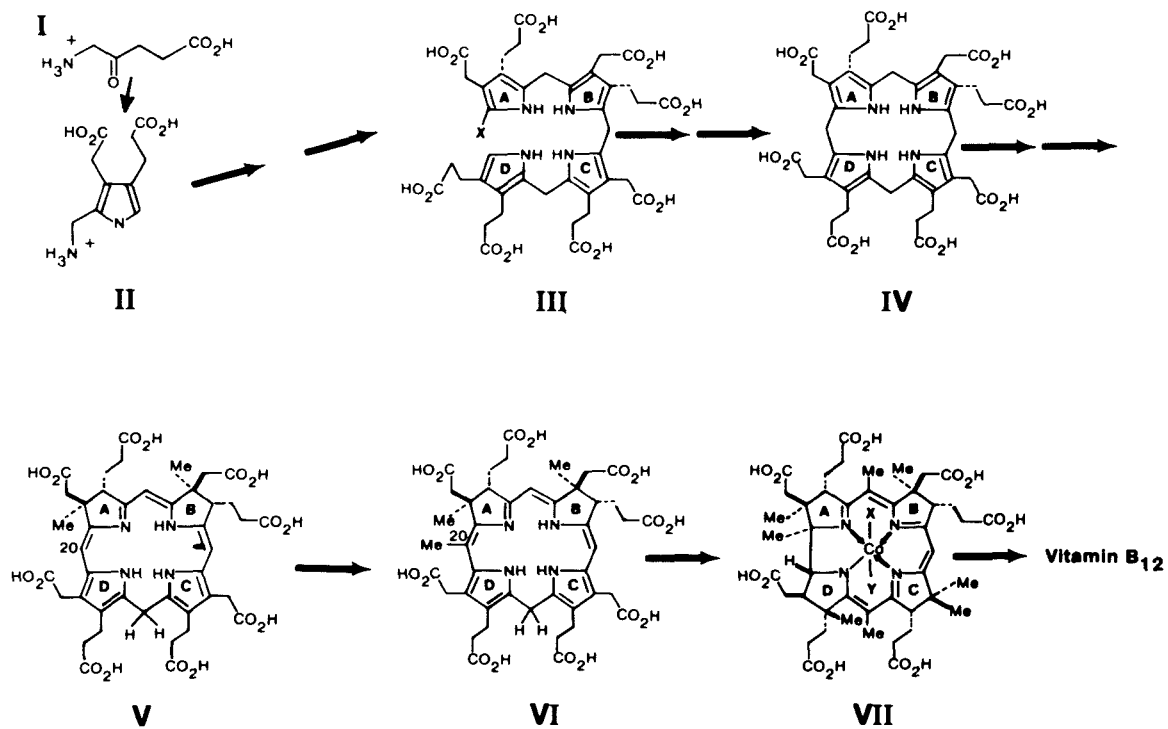


Figure 3. Selected Steps in the Biosynthesis of Cyanocobalamin.

nite (Fuller's Earth), sodium montmorillonite (bentonite) or resins; eluted, purified by extraction, and precipitated as the copper- or zinc-cyanocobalamin complexes. Recrystallization is often from acetone. With commercial improvements of yields, the price dropped from about \$800 to \$18 a gram. A brief summary of patents is also included in reference 12. For formulation into animal feed and vitamin supplements, cyanocobalamin is available as low, medium or high concentrates, as crystals and adsorbed on such carriers as resins (*cf.* section 6.2, Stabilization).

1.5 Nutrition, Physiology and Medicine

Occurrence in food has been summarized.²⁷ Because plants lack corrinoids, either vitamin B₁₂, or related compounds, must be obtained by animals from intestinal bacteria, milk, meat, fish or voluntary or involuntary coprophagy.

Human daily dietary allowances²⁸ in μg are: Infants to 1 year, 1.5; children 3; males 3; females 3, and pregnant or lactating females, 4. Much of the human requirement is met by intestinal bacterial synthesis. In lower animals, the requirement for cyanocobalamin depends on the species, and is about 2 to 20 μg per kg of diet.

Once ingested by man and other mammals, dietary cyanocobalamin is bound by intrinsic factor and R binder proteins which are located in the gastric and intestinal juices.²⁹ The intrinsic factor-cyanocobalamin complex appears to join, at neutral pH and with calcium, to a third protein in the small intestine, membrane-bound intrinsic receptor. Cyanocobalamin is transported in blood attached to a fourth protein, transcobalamin II and a binder, transcobalamin I. The binding capacity is determined by the number of such sites. The binding capacity measurements have been used diagnostically to differentiate between different diseases, such as leukemia and non-leukemic leukocytosis. In microorganisms, binding of cyanocobalamin initially is to the outer membrane. Transfer into the interior of the cell requires a proton motive force and, perhaps, an additional carrier.

In humans, cobalamin content of normal tissues, in ng/g are as follows,³⁰ with the percentage of cyanocobalamin in parenthesis; liver, 1050 (0%); kidney, 134 (0%); spleen, 63 (0%); brain, 81 (0%); pituitary, 230 (0%); bone marrow, 13 (2%), leucocytes, 4 (4%); plasma, 0.4 (2%); erythrocytes, 0.2 (6%); bile, 18 (7%) and cerebrospinal fluid, 0.02 (10%). The rest of the cobalamins are the methyladenosyl- or hydroxo- forms, as determined by chromatography and bioautography.

Vegetarians, as expected, frequently show abnormally low concentrations of cobalamins in their body fluids, as do some individuals ingesting megadoses (more than 2 grams a day) of vitamin C. Other conditions and diseases producing low concentrations in human serum include malabsorption because of pregnancy, iron deficiency, primary folate deficiency, cancer (especially multiple myeloma), aplastic anemia, hereditary absence of transcobalamin I and use of oral contraceptives. In lower animals, cobalt may be deficient in the soil of the region, and lead to vitamin B₁₂ deficiencies unless supplemented.

Deficiency symptoms in man³¹ include pernicious anemia, which results from a diminution in the reduction of ribonucleotides to deoxyribonucleotides. Large red blood cells may form which contain immature nuclei. The spinal cord may degenerate. Insufficient cyanocobalamin is often indicated by the excretion of methylmalonate into the urine, due to impaired conversion to succinyl coenzyme A (*cf.* Section 1.62, Reactions, *In Vivo*). In lower animals,³² deprivation of cyanocobalamin is first seen in the young by poor growth and then in all animals by fewer red blood cells, less plasma protein, higher non-protein nitrogen values in serum, less glucose in serum and, eventually, by higher mortality rates.

Typically, patients testing below 200 ng cyanocobalamin per liter (148 pmol/L) are retested for vitamin B₁₂ and folate.³³ Treatment usually includes cyanocobalamin, with or without added folic acid.

1.6 Reactions of Cyanocobalamin

1.61 *In Vitro*

The cyanide group in vitamin B₁₂ can be removed by photolysis or reduction³⁴ to give aquocobalamin (H₂O-Co $\ddot{\text{N}}$) which, in turn, can react with various acids to form the bromide, chloride, cyanate, nitrate, sulfate and thiocyanate. As expected, cyanide can reverse these reactions to form vitamin B₁₂. Potassium cyanide added to aqueous cyanocobalamin displaces the nucleotide giving the purple dicyanide.

The addition of acids, results in the expected hydrolysis of amide, ester and glycosylamine bonds, as well as protonation (*cf.* Section 4.4, Ionization).

Cyanocobalamin reacts with chlorine in mild acid to form a lactone [8-hydroxy- α -(5,6-dimethylbenzimidazolyl) cobamic acid *abdeg* pentamide-*c*-lactone].

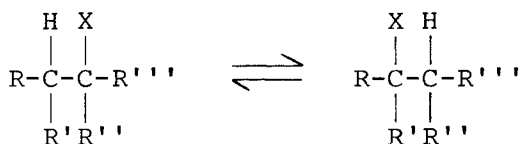
Reduction (*cf.* Section 5.42, Polarography) of vitamin B₁₂ [cob(III)alamin], by such compounds as cysteine, results in the brown-orange to gray-green intermediate cob(II)alamin or B_{12r}. Biological reductants appear to be thiol or flavin compounds. Further reduction of B_{12r} gives B_{12s} or cob(I)alamin. B_{12r} can disproportionate to B_{12s} and aquo-B₁₂. B_{12s} in aqueous acid decomposes to cob(II)alamin and molecular hydrogen. B_{12s} is one of the most powerful nucleophiles known.³⁵ It reacts with BrCN to give B₁₂-CN, with H₃CI or dimethylsulfate to form B₁₂-CH₃, with Br-C \equiv CH to yield B₁₂-C \equiv CH, with HC \equiv CH or H₂C=CHBr to produce B₁₂-CH=CH₂, with ethylene oxide or ClCH₂-CH₂OH to give B₁₂-CH₂-CH₂OH, with ethyleneimine to yield B₁₂-CH₂-CH₂-NH₂, with H₂-C=CH-CO₂H to give B₁₂-CH₂-CH₂-CO₂H and with acetyl chloride or acetic anhydride to give B₁₂-CO-CH₃.³⁶

Immobilized derivatives of cobalamins have been prepared³⁷ and used to purify

cobalamin-dependent enzymes and binding proteins.

1.62 *In Vivo*

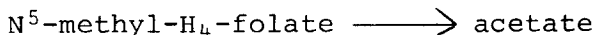
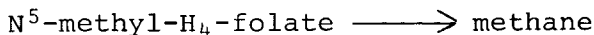
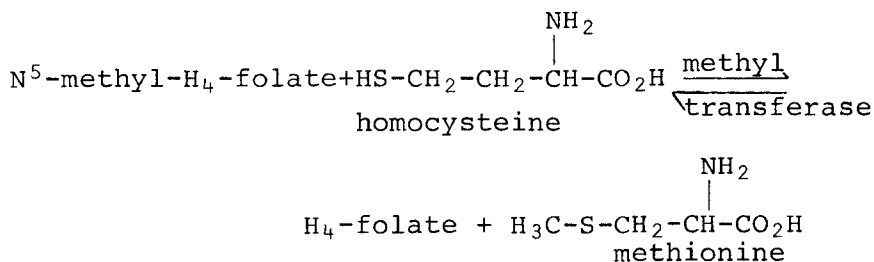
Vitamin B₁₂ is converted to the orange-yellow coenzyme by replacement of the cyanide group, attached to the central cobalt atom, by 5'-deoxyadenosine. Reactions involving coenzyme B₁₂ can be generalized to be of the type shown below, with the exception



of the nucleotide reductase conversion. A hydrogen migrates from one carbon atom to an adjacent one with the concomitant movement of an acyl, alkyl or electronegative group (X) from the adjacent carbon atom to the one to which the hydrogen was originally bound.³⁸ The less branched structure has the greater thermodynamic stability.³⁹

Reactions involving methylcorrinoid as the intermediate, as shown below, appear to also require folic acid (pteroylglutamic acid) in the form of tetrahydrofolate.

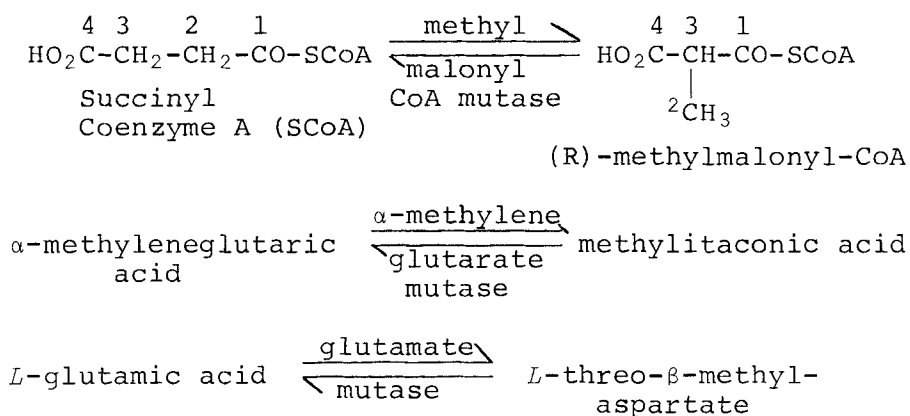
Transmethylation



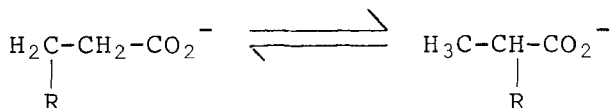
The reaction appears to involve a Co-CH₃ complex as intermediate.^{40,41} Curiously, enzyme-bound⁴² [¹⁴C]-methyl-B₁₂ is less light-sensitive than free [¹⁴C]-methyl B₁₂.

Methane can be formed by microorganisms from methanol, carbon dioxide, formate or and formaldehyde.

Carbon Skeleton Rearrangements



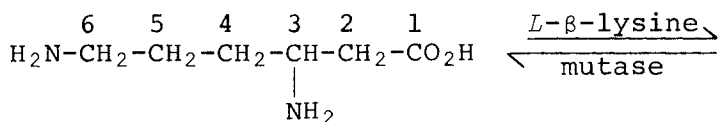
The reactions catalyzed by these enzymes are similar in that a substituent group is transferred between the α and β position of a propionate moiety, while a hydrogen atom is moved in the opposite direction.^{43,44} These studies involved ¹⁴C-labeled compounds and D₂O.⁴⁵



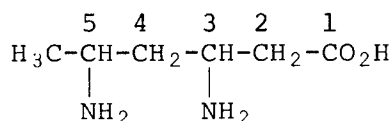
In such conditions as pernicious anemia, the conversion of (R)-methylmalonyl-CoA is diminished, and relatively large concentrations of methylmalonic acid are found in the urine (which may be used as an indicator of cyanocobalamin deficiency⁴⁶). This reaction is the only coenzyme B₁₂-dependent rearrangement⁴⁷ occurring in higher animals and man as well as in bacteria. It enables propionyl-CoA,

originating from branched fatty acids and amino acids, to be metabolized via the citric acid cycle.⁴⁸

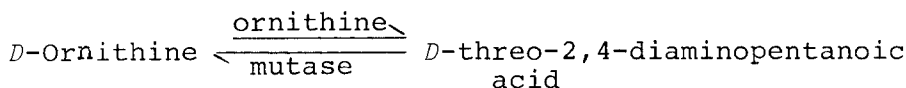
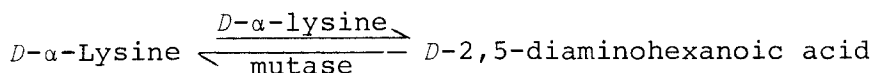
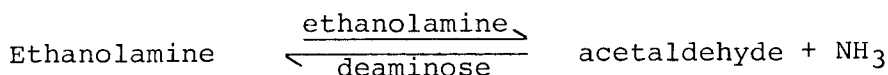
Amino Group Migrations



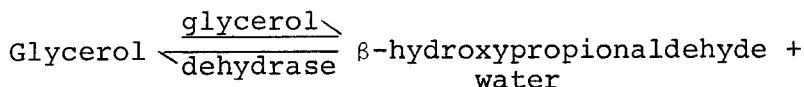
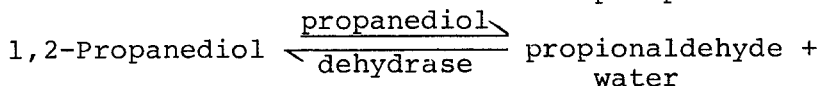
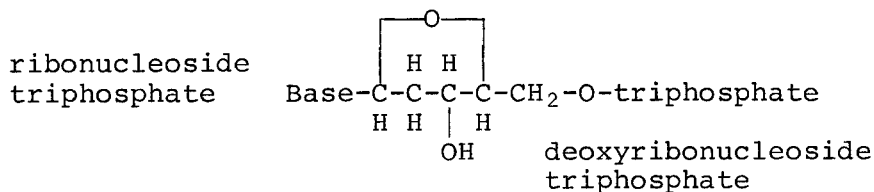
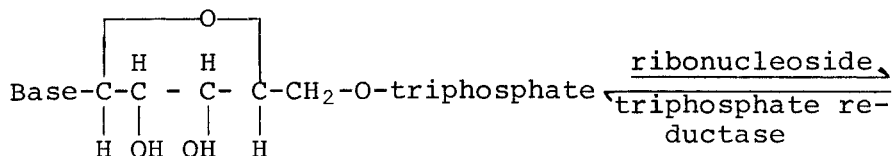
L-β-lysine



L-erythro-3,5-diaminohexanoic acid



The exchange of the amino group and hydrogen between adjacent atoms in the ethanolamine deaminase (EC 4.3.1.7), lysine mutase and ornithine mutase reactions is similar to the previously-discussed carbon skeleton rearrangements. The migrating hydrogen is transferred stereospecifically from substrate to coenzyme. Tritium is transferred from B₁₂ coenzyme specifically labelled in the 5'-methylene position of the 5'-deoxyadenosyl moiety to acetaldehyde by ethanol deaminase, to 3,5-diaminohexanoate by β-lysine mutase, and to 2,5-diaminohexanoate by α-lysine mutase. Addition of substrate to ethanolamine deaminase-B₁₂ coenzyme gives rise to electron spin resonance signals characteristic of a radical.⁴⁹ The bound enzyme was found to be 95% in the form of cob(II)alamin.⁵⁰

Net Reduction

Diol dehydrase (EC 4.2.1.28) and glycerol dehydrase (EC 4.2.1.30) catalyze the transfer of a hydrogen from the C-1 position of the substrate to the C-2 position, while the hydroxyl group on C-2 moves to C-1 of the product.⁵¹ This is analogous to the preceding amino group rearrangements. Stereochemical results and cobalt involvement in these reactions have been discussed.⁵² Ribonucleotide reduction to 2'-deoxyribonucleotide is required for deoxyribonucleic acid synthesis and, eventually, growth and red blood cell production. The enzyme, ribonucleotide triphosphate reductase, replaces the hydroxyl group at C-2' of a ribonucleotide by hydrogen in an S_N1 type of reaction. The hydrogen was found to be carried by the B₁₂ coenzyme and cobalt-coenzyme bond cleavage occurs.⁵³ The source of the hydrogen was reduced nicotinamide adenine dinucleotide phosphate, which was transferred to a low molecular weight dithiol protein. Electron spin resonance studies show the presence of B₁₂r signals, together with signals ascribable to organic free-radicals.⁵⁴

2. Physical Properties of Crystalline Cyanocobalamin

2.01 Single Crystal X-Ray Diffraction

The three-dimensional structure was obtained originally by Dorothy Crowfoot Hodgkin and co-workers^{55,56,57} by means of single crystal X-ray diffraction. Partly as a result of this work, she was awarded the Nobel prize for Chemistry, in 1964.

Crystals of cyanocobalamin were grown in water: Molecular weight 1752; probable formula, $C_{63}H_{88}N_{14}O_{14}Co \cdot P \cdot 22H_2O$. The structure is shown in Figure 4. The agreement factor, R, is 26%. The structure of B₁₂-5'-phosphate, the biosynthetic precursor, was similar⁵⁸ to that of B₁₂ found by Hodgkin and coworkers.⁵⁹ However, slight differences in rotations of the α -glycosidic bonds, two acetamide groups and two water molecules were apparent. The structure of the 5,6-dimethylbenzimidazolylcobamide coenzyme was also determined⁶⁰ to a spacing limit of 0.9 Å.

In aqueous solution, the average orientation of the deoxyadenosyl moiety is significantly different from that found in the crystalline state.⁶¹ The corrin macro-ring is thought to exist as photostable and photolabile isomers. Isomerization depends on pH and solvents, and is reversible. In an epimer⁶² at C-13, the propionamide group can orient itself up or down, following different puckering of the corrin ring. Little change was found to be apparent⁶³ in molecular packing. The ratio of neo to normal form is 3 or 4 to 1.

The structure of an impurity in commercially available cyanocobalamin was studied by X-ray crystallography.⁶⁴ Base was lacking in what amounts to 0.08% of the usual form.

Neutrons⁶⁵ can be used to determine molecular structure similarly to X-rays. However, neutrons are difficult to generate, cobalt was seen as a "light" atom in the neutron Fourier diagram because all nuclei scatter neutrons within approximately the same order of magnitude, and hydrogen

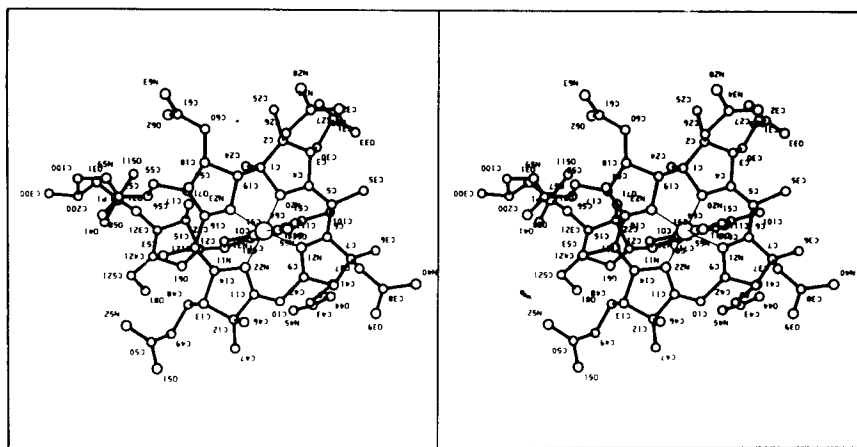
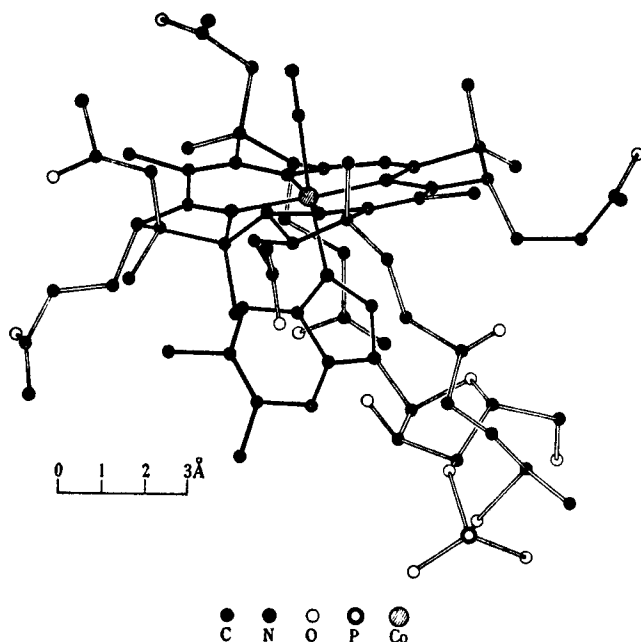


Figure 4. Stereoview of Cyanocobalamin, upper portion, lower portion, Enlargement Showing Position of the Atoms. (From Reference 58, by permission).



cannot be neglected in the vector diagram, thus requiring more reflections. Because of these obstacles neutron structure elucidation is rarely used. The three-dimensional X-ray and neutron analyses of cyanocobalamin gave similar results.⁶⁶

2.02 X-ray Powder Diffraction, Spectrometry and Activation Analysis

Figure 5 is the powder X-ray diffraction pattern⁶⁷, as obtained using a Philips powder diffraction unit emitting $\text{CuK}\alpha$ radiation at 1.54 Å. With the aid of a scintillation counter detector, the sample was scanned and recorded from approximately 2 to 36 degrees (2θ).

The cobalt complex in cyanocobalamin was studied by X-ray photoelectron spectroscopy⁶⁸, to give peak separation and binding energies. Bombardment by 8eV ^3He gave rise to X-ray emissions which can be used to detect cobalt with a 1 to 10 ng limit of detection.⁶⁹

2.03 Mass Spectrometry

Because conventional mass spectroscopy techniques using volatilization induce thermal strain in cyanocobalamin, a gentler mode of ionization was used. Field desorption mass spectrometry, utilizing an electrostatic field of 10^7 to 10^8 V/cm to cause but one electron to be lost, is ideal for the analysis of vitamin B_{12} . Figure 6 is the field-desorption mass spectrum⁷⁰ of cyanocobalamin using laser heating⁷¹ to increase ionization efficiency and sensitivity. The molecular ion was apparent at m/e 1354.567. The ion at m/e 1295.530; elemental composition $\text{C}_{61}\text{H}_{83}\text{N}_{13}\text{O}_{13}\text{CoP}$, may originate from the loss of acetamide. Dehydration may produce the ion at m/e 1318.546; elemental composition $\text{C}_{63}\text{H}_{84}\text{N}_{14}\text{O}_{12}\text{CoP}$. The base peak at m/e 914.445 agrees with an ion with the elemental composition $\text{C}_{45}\text{H}_{65}\text{N}_{11}\text{O}_6\text{Co}$, which may arise from the splitting of the nucleotide side chain.⁷² Compounds related to cyanocobalamin and depletion of ^{13}C during the biosynthesis of vitamin B_{12} were studied by this technique using but 1 μg of material.⁷³ Mass fragmentography is discussed in Section 5.8.

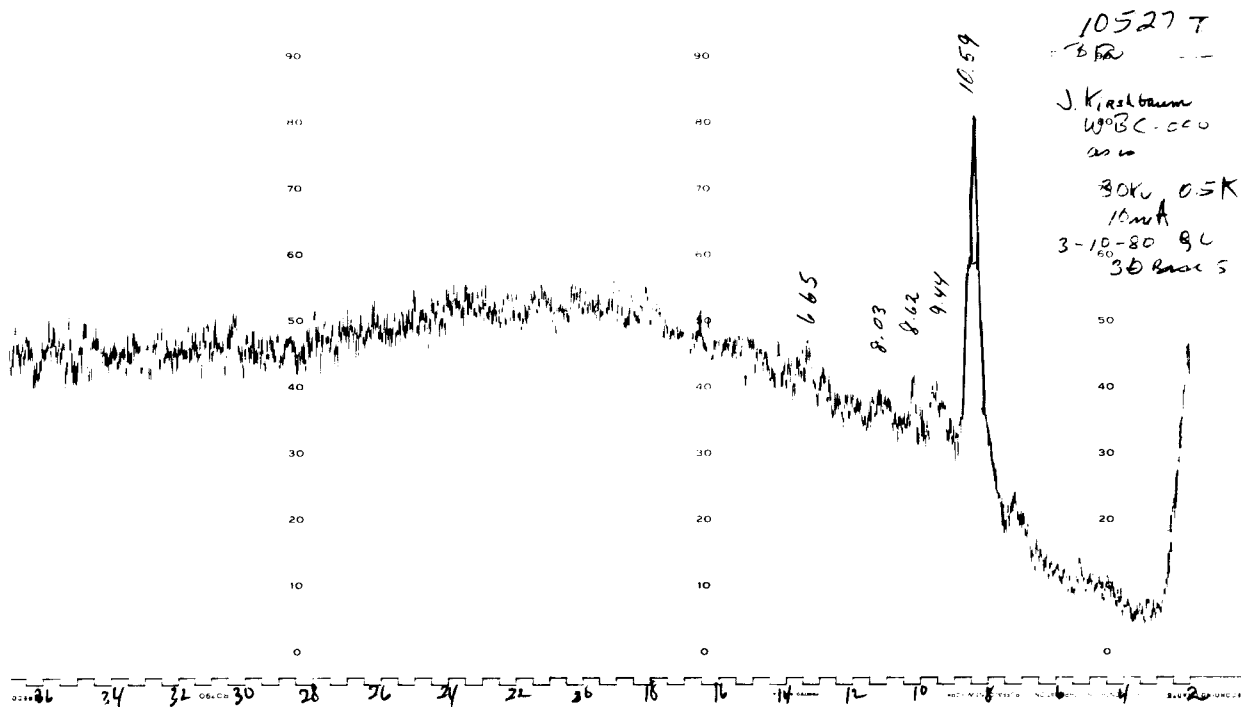


Figure 5. Powder X-ray Diffraction Pattern of Cyanocobalamin.
See text for details.

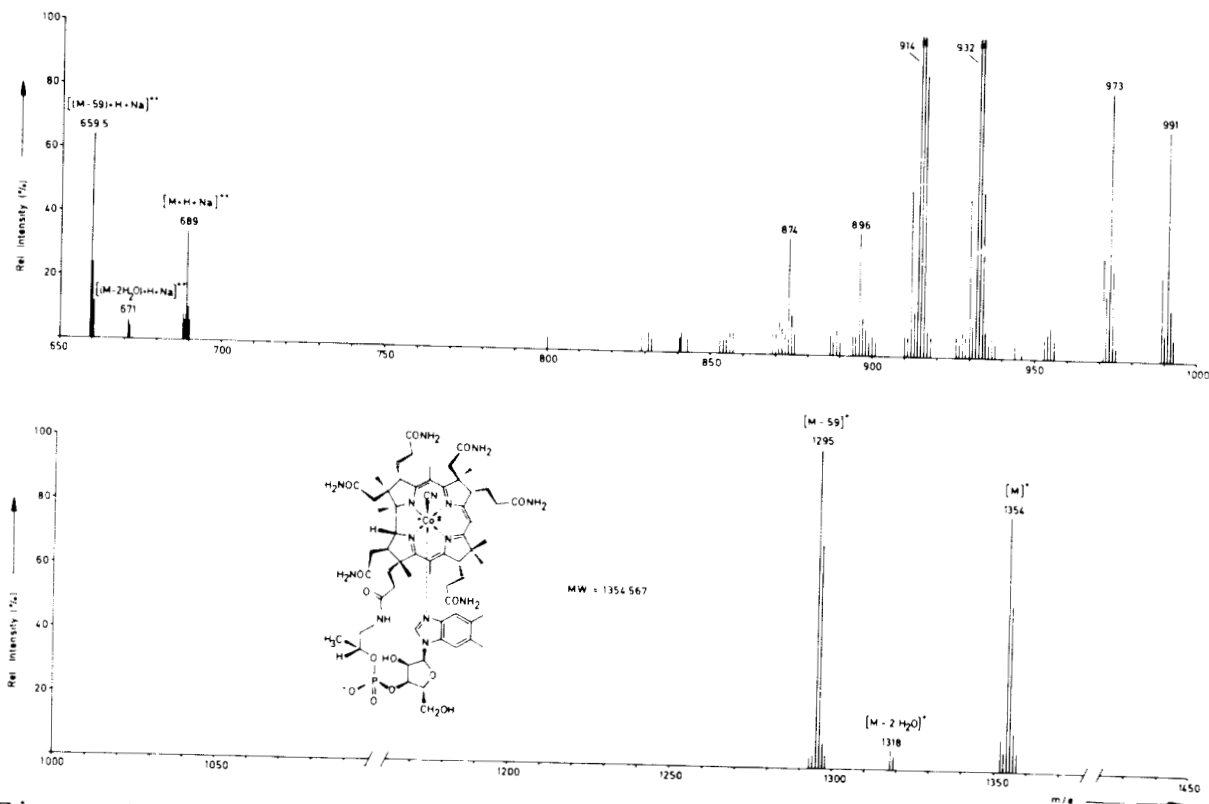


Figure 6. Field-Desorption Mass Spectrum of Cyanocobalamin, (supplied by H.-R. Schulten) See text for details.

2.04 Magnetic Susceptibility

Cyanocobalamin is diamagnetic^{74,75,76} in solution and the solid state. The molar susceptibility, χ_m is $-750 \pm 100 \times 10^{-6}$ and the Bohr magneton (μ) value⁷⁷ is approximately 1.21. An impurity present in some preparations, with a concentration $<0.1\%$, is paramagnetic, and has lead to error in some determinations.⁷⁷ The related aquo- and adenosylcobalamin are also diamagnetic. The triple charge of the cobalt is neutralized by the negative charges of the cyanide ion, one of the corrin ring nitrogens and the phosphate of the nucleotide.

2.05 Mössbauer Spectrometry

Mössbauer spectra are characteristic of the highly conjugated nature⁷⁸ of cyanocobalamin. A doublet was visible at 77°K using⁵⁷ Co-labeled vitamin B₁₂. Emission Mössbauer spectra of cyanocobalamin, with and without the 5,6-dimethylbenzimidazole base, were studied.⁷⁹ The spectra may be explained on the basis of considerable delocalization of electrons onto the corrin ring.

2.06 Thermal Analysis

From 20°C to 140°C , water was found to be lost in a single step. Visible⁸⁰ during thermal gravimetric analysis, was a sharp symmetrical peak at 135°C . Between 140°C and 145°C an approximately 2% loss in mass was correlated with removal of the cyanide group using ultraviolet and infrared spectrometry (loss of 2137 cm^{-1} absorption), and quantitative determination of cyanide using a ion-selective electrode (*cf.* section 5). Four endothermic effects were found for the black colored residue at about 230°C , 280°C , 390°C and 520°C . At approximately 235°C , the spectrum up to 1200 cm^{-1} indicates cleavage of the bond between 5,6-dimethylbenzimidazole and the corrinoid system. At 800°C , the remaining blue-violet mixture of phosphorus and cobalt oxides, if calculated as P_2O_5 and Co_2O_3 is equal to 11.3% of the initial cyanocobalamin content (theoretical value, 11.1%). The thermogravimetric weight loss appeared to be a zero order reaction with an activation energy of $26.7 \pm 1.9\text{ kcal/mole}$.

In another study⁸¹ using a different preparation of cyanocobalamin, an endotherm occurred at 97°C, indicating variability of the vitamin B₁₂ since this peak is also ascribed to the loss of water. Endotherms at approximately 240°C and 250°C were accompanied by melting and decomposition, precluding analysis of purity by differential scanning calorimetry.

Following the U.S.P. procedure⁸² for class 1A compounds, the melting range of cyanocobalamin was determined. Decomposition started at 205°C and appeared to end at 209°C, in good agreement with the Merck index¹² value of darkening of the crystals at 210°C.

2.07 Microscopy and Crystal Type

Microscopically,⁸¹ three sizes of particles are visible; 10 x 10 μ , 150 x 150 μ and 500 x 500 μ , in a commercial preparation of cyanocobalamin. The largest particles are not agglomerated. For good crystals the refractive indices are α = 1.616, β = 1.652 and γ = 1.644. The system is orthorhombic and the crystal habit prismatic. When crystals of vitamin B₁₂ are grown in water,⁵⁷ the unit cell constants are; a = 25.33 Å, b = 22.32 Å and c = 15.92 Å, space group P2₁²₁2₁; n = 4; density 1.29 (measured in a wet mixture of acetone and bromobenzene; calculated value 1.292). Here, the crystals measured 0.1 to 0.4 mm in each dimension, with well defined faces, usually {110} and {111}.

Cyanocobalamin was imaged using field ion microscopy.⁸³ The vitamin was added to platinum, which was electrodeposited on the tungsten specimen tip prior to the gradual removal of surface layers by controlled field evaporation. A few two-fold symmetrical patterns are visible.

2.08 Surface Area

As measured by gas adsorption,⁸¹ the surface area of one commercial preparation of cyanocobalamin is 0.57 m²/g.

2.09 Hydration

The number of molecules of water per mole of water-grown crystals of cyanocobalamin is 22; 18 are present per molecule of air-dried vitamin.⁵⁷ The unit cell contains water in channels, at such a high concentration that single crystal X-ray studies show that these pools of water may have some freedom of movement. Elemental and vapor phase chromatographic analyses of a commercial preparation (*cf.* section 5.1) gave values of approximately 5 moles of water per mole of vitamin B₁₂; indicating variability of the water content.

2.10 Polymorphism

Different crystal forms of cyanocobalamin are likely to exist since X-ray crystallography studies show that contacts between individual molecules can vary, especially if the water content varies. In addition, the corrins can exist as interconvertible isomers⁶¹ (*cf.* section 2.1).

3. Spectrometry of Cyanocobalamin in Solution

3.1 Nuclear Magnetic Resonance Spectrometry (NMR)

3.11 ¹H-NMR

Figure 7 is the 360 MHz spectrum of 10 mg cyanocobalamin per mL deuterium oxide, pH 6, at 25°. The assignments⁸⁴ for the chemical shifts (δ) in ppm, relative to 2,2-dimethyl-2-silapentane sulfonic acid measured at 360 MHz, and the longitudinal relaxation rate (T₁), measured at 100 MHz, are given below. The location of the atoms is shown in Figure 1.

<u>δ (ppm)</u>	<u>T₁ (ms)</u>	<u>Assignment</u>
0.448	55	C-20
1.187	144	C-47
1.253 (doublet J _{1H} = 6Hz)	195	methylene C of isopropanolamine
1.380	132	
1.863	116	
2.253	203	C-35 and C-53
2.536	144	B-10 and B-11
2.570		B-10 and B-11

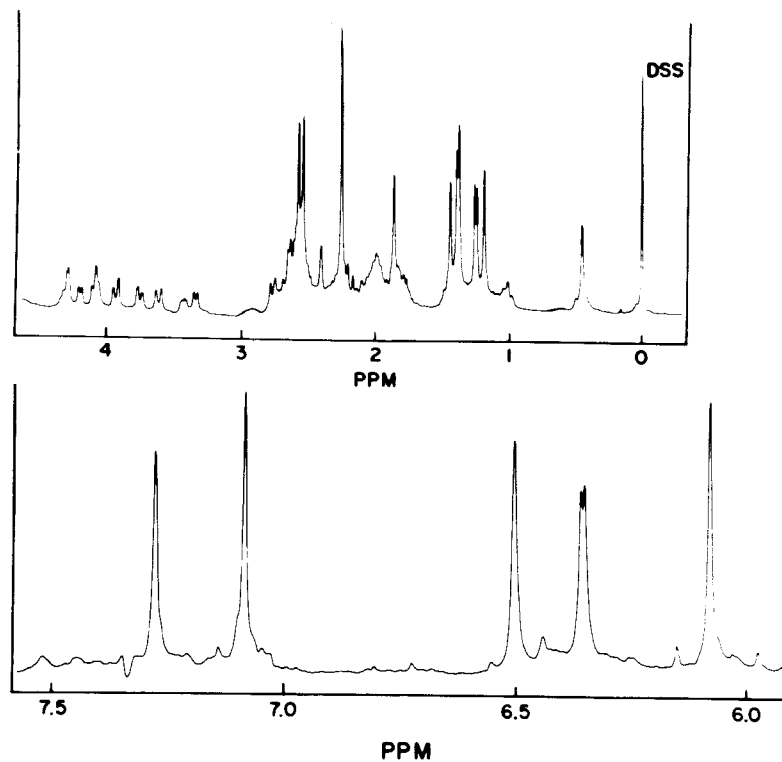


Figure 7. Low Field (6.0-7.5) and High Field (0-4.5) portions of the 360 MHz ^1H -NMR Spectrum of Cyanocobalamin, courtesy of A. Nath.

δ (ppm)	T_1 (ms)	Assignment
6.081	205	C-10
6.349 (doublet)	239	R-1
6.503	294	B-4
7.081	298	B-7
7.275	335	B-2

These results agree with the 220 MHz assignments^{85,86,87,88}. The spectrum was found to be dependent on pH from 2 to 9 (*cf.* Ionization, section 4.4).

3.12 ^{13}C -NMR

High-resolution Fourier transform NMR at 15.08 MHz was used to observe the proton-decoupled, natural-abundance ^{13}C spectrum of 0.024 M cyanocobalamin in water,⁸⁹ Figure 8. Assignments were made using splittings arising from ^{13}C - ^{31}P coupling, chemical shift comparisons, off-resonance single frequency proton decoupling and partially-relaxed Fourier transform spectra. See Figure 1 for the locations of the carbon atoms.

Assignment	Chemical Shift
Corrin ring carbons	
5	85.9
15	89.3
10	98.5
1	108.1
19	118.3
2	134.1
3 }	{ 137.5
8 }	{ 139.3
13 }	{ 142.0
7 }	{ 145.1
12 }	{ 145.9
17 }	
18	—
α -Ribazole and isopropanolamine carbons	
2	51.6
9	56.7
8	58.4
5	60.5
6	63.5
4	76.9
7	82.0

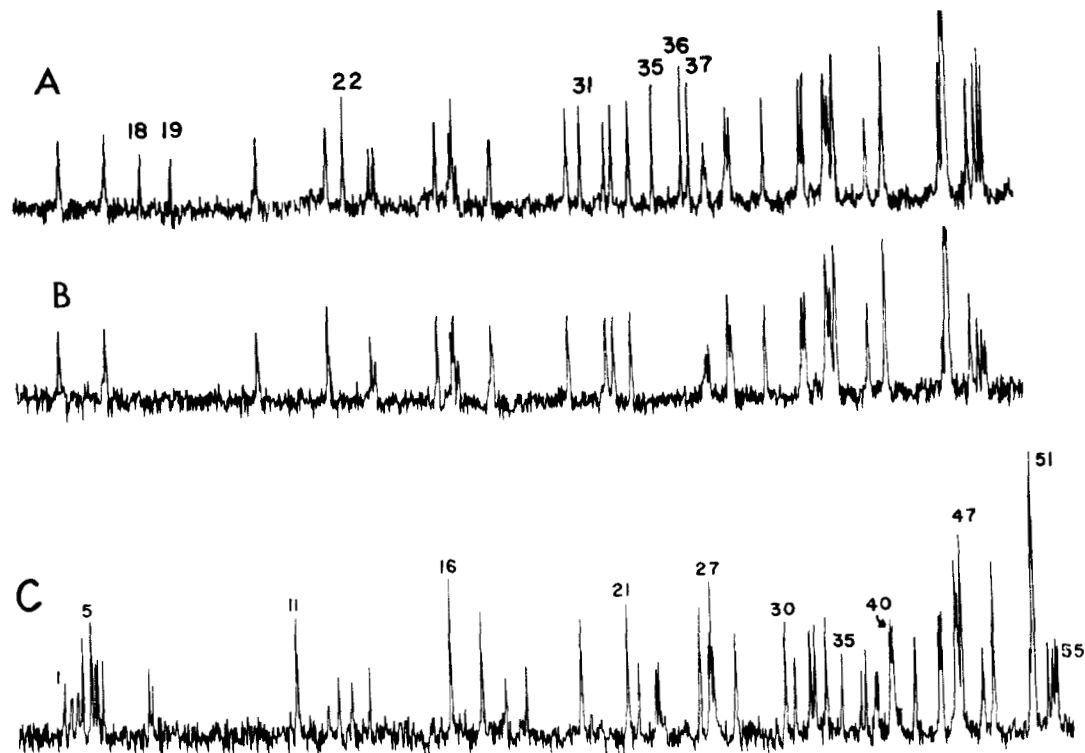


Figure 8. ^{13}C -NMR Fourier Transform Spectrum of 0.024M Cyanocobalamin at 15.08 MHz. A, Normal Spectrum of upfield portion of cyanocobalamin at 61° using 4096 points in the time domain and 16,284 scans, B, partially relaxed Fourier transform spectrum, $\tau=1.02$, to determine non-protonated carbons of the corrin ring, and C, completely proton decoupled ^{13}C spectrum. See reference 89 and text for details.

<u>Assignment</u>	<u>Chemical Shift</u>
1'	106.2
4'	111.2
2'	120.0
3'	120.3
5'	132.5
CH(doublet)	124.3
CH ₂	147.8

¹³C-NMR spectra have also been obtained for related compounds.^{89,90,91}

3.13 ¹⁵N-NMR

Although ¹⁵N produces sharp resonances because of its spin of 1/2, its NMR sensitivity is 1.04×10^{-3} that of the proton. In addition, it is less abundant (0.37%), giving a sensitivity of 3.8×10^{-6} that of the proton. These difficulties can be partially overcome by placing large sample volumes, 15-30 mL, in 25-mm. diameter tubes. A spectrum at 18.25 MHz, using a Bruker WH-180 Fourier-transform, superconducting spectrometer, of cyanocobalamin enriched (2.4%) in ¹⁵N showed seven resonances⁹² at 256.8 to 268.2 ppm. The external shift reference is 0.1 M D¹⁵N₂O₃ in deuterium (D) oxide. These amide nitrogens resonate in the same region as other large biological molecules. The ring nitrogens were not observed. Perhaps the lack of signal intensity was due to coupling to cobalt, nuclear Overhauser effects and long relaxation times.

3.14 ³¹P-NMR

A Nicolet NTC 150 widebore superconducting spectrometer was operated at 60.7 MHz. The external chemical shift reference was 85% phosphoric acid (0.0 Hz). At a concentration of 0.0012M, the chemical shift was 4.79 Hz⁹³ with a line width of 5.7 Hz at pH 8.1.

3.2 Electron Spin Resonance Spectrometry

Lack of electron spin resonance (ESR) signals both in frozen solution and the crystalline solid⁹⁴ demonstrate that the central cobalt atom in cyanocobalamin is trivalent and diamagnetic. ESR has

been applied⁸⁷ to the study of vitamin B₁₂-dependent enzymes and to derivatives of cyanocobalamin⁹⁵ (cf Section 1.42, reactions of cyanocobalamin, *in vivo*) characteristics of these spectra are $g_{\perp} = 2.25$, $g_{\parallel} = 2.003$, $A_{\parallel}(\text{Co}) = 196 + 2 \times 10^{-4} \text{ cm}^{-1}$ and $A_{\perp} = 15.8 + 0.5 \times 10^{-4} \text{ cm}^{-1}$, with the hyperfine and superhyperfine splitting sensitive to the nature of the axial ligands.⁹⁶ Cob(II)alamin is paramagnetic and cob(I)alamin, as expected, is diamagnetic.

3.3 Infrared Spectrometry

Figure 9 shows the infrared spectrum of cyanocobalamin recorded as a KBr pellet using a Perkin-Elmer infrared spectrophotometer. Below are the interpretations of the various absorptions.⁹⁷

<u>Absorption (cm⁻¹)</u>	<u>Assignment</u>
3400	O-H
3200 broad	N-H
2950	C-N
2060	C \equiv N
1660	C=O
1570	{ C=C
1490	
1060 broad	PO ₄ ⁻

Infrared spectra of cyanocobalamin and such related compounds as adenosylcobalamin and aquocobalamin were compared in another study.⁹⁸ The spectra closely resemble each other.

3.4 Raman Spectrometry

Raman spectra of aqueous solutions of cyanocobalamin at concentrations of $\sim 10^{-4} M$ were obtained using Ar⁺ and He/Ne lasers. The intensity of a strong band at 1502-1504 cm⁻¹ depends on the wavelength of the exciting radiation used,^{99,100} confirming that these are resonance Raman spectra, and are due to the corrin ring.¹⁰¹ A study of vitamin B₁₂ and related compounds indicated¹⁰² that the $\sim 1500 \text{ cm}^{-1}$ band was due to π - π^* transitions. The conformation of the corrin ring and

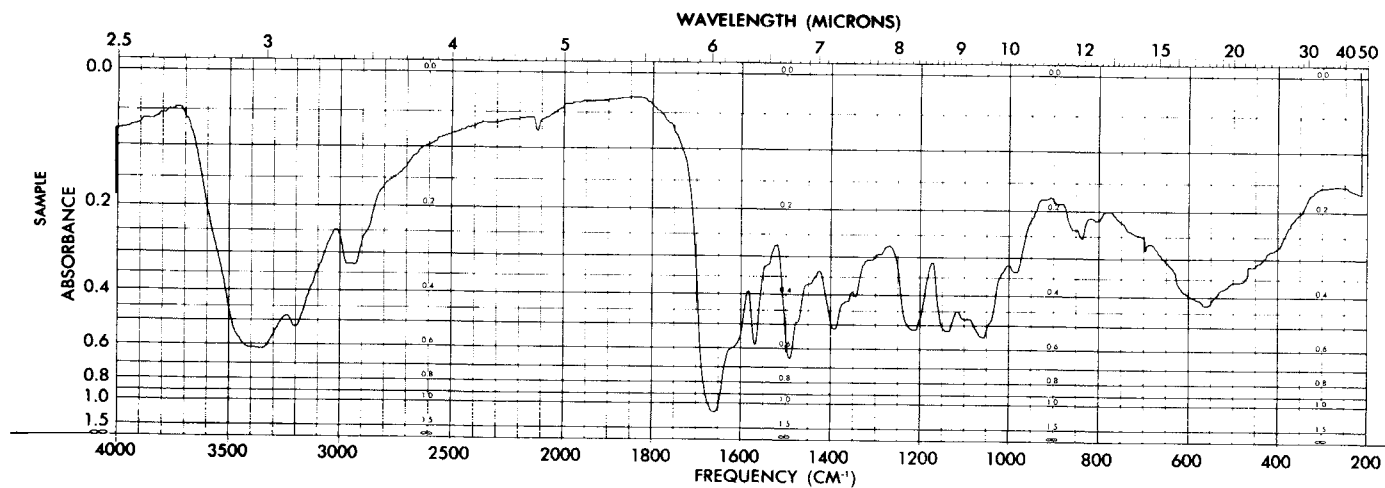
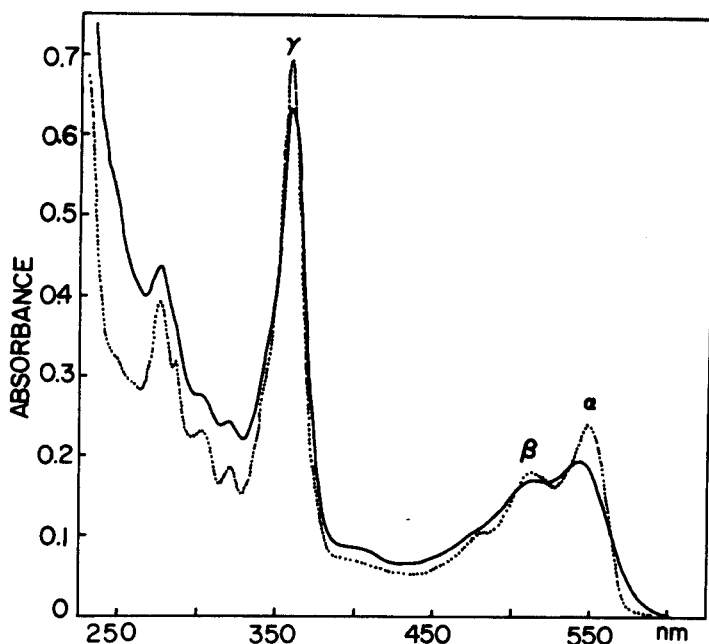


Figure 9. Infrared Spectrum of Cyanocobalamin.
See text for details.

the degree of coordination of the cobalt atom was also studied using Ar and Kr lasers.¹⁰³ The effect on the spectra of other water-soluble vitamins was studied.¹⁰⁴ Only riboflavin had a significant effect on the 1504 cm^{-1} signal.

3.5 Absorption (Visible and Ultraviolet Spectrometry)

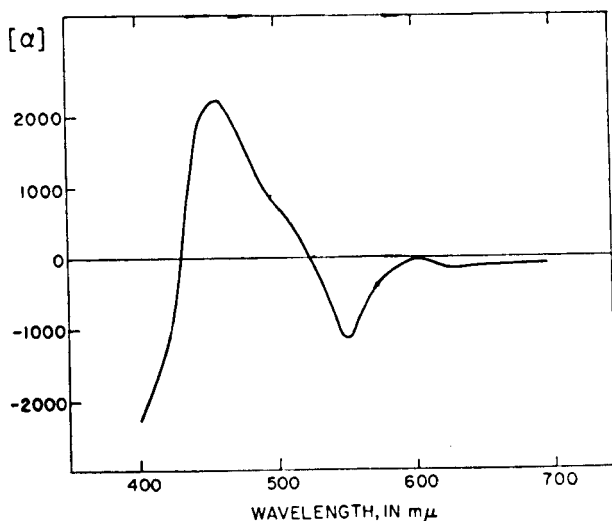
The figure below shows the absorption spectrum at 298°K (—) and 77°K(---) of cyanocobalamin in ethanol. These spectra, courtesy of P.-S. Song,¹⁰⁵ illustrate the classical α , β and γ "Soret" bands characteristic of porphyrins and chlorophylls. The electronic spectra of corrins originate primarily in the 14 π -electron system of the conjugated corrin and secondarily in the cobalt ligand.^{106,107} The cyanide group above the plane of the corrin ring perturbs the π -electron system.¹⁰⁸ Changes in the oxidation state of the central metal are accompanied by considerable changes in the spectrum. The spectrum varies with solvent (as shown below),¹⁰⁹ pH⁸⁴ and temperature.^{109,110}



Solvent	Absorption (λ) nm	Absorbance Relative to λ band (~ 360 nm)
Dimethylsulfoxide	360	1.00
	519	0.29
	546	0.33
Water	360	1.00
	518	0.27
	549	0.30
Methanol-	360	1.00
Ethanol(1:4)	518	0.28
	547	0.31

3.6 Optical Rotatory Dispersion and Circular Dichroism Spectrometry.

Cyanocobalamin has 15 asymmetric carbons in addition to asymmetry around the cobalt atom. The figure below shows the optical rotatory spectrum of 0.05% aqueous vitamin B₁₂, modified from reference 111.



Such a major change as substitution of cyanide by water coordinating to the cobalt atom results in only minor spectral differences, indicating a limitation of this technique. Spectral differences exist between the Co(III) and the Co(II) forms of cyanocobalamin. Circular dichroism studies show a marked solvent effect,¹⁰⁹ as shown on the next page.

Solvent	Wavelength (λ) nm	Dichroism ($\Delta\epsilon$)
Dimethylsulfoxide	310	-6.0
	519	+11.5
Water	360	-8.6
	518	+15.1
Methanol-	360	-8.9
Ethanol (1:4)	518	+13.3

The spectrum obtained at room temperature is inverted at the approximately -180° temperature of liquid nitrogen, indicating a change in conformation ($\Delta H = \sim 2-3$ Kcal).

3.7 Fluorescence Spectrometry

Although cyanocobalamin has little natural fluorescence,¹¹² fluorescence can be induced^{113, 114} by photolysis or cyanolysis of the carbon-cobalt bond (cryptofluorescence). Such a technique, coupled with sensitive, low-noise microprocessor circuits should be useful in developing assays for cyanocobalamin.

4. Bulk Solution Properties

4.1 Intrinsic Dissolution Rate

The intrinsic dissolution rate was determined¹¹⁵ after compressing powder under 1500 PSIG using 3/8" diameter, disc-shaped dies. The surface area was 0.713 cm^2 . In one liter of water at room temperature, agitated at a rate of 50 rpm, the intrinsic dissolution rate of cyanocobalamin is $0.275 \text{ mg min.}^{-1} \text{ cm}^2$, using ultraviolet spectrometry at 361 nm (*cf.* section 5.2, spectrometry).

4.2 Solubilities in Aqueous and Non-Aqueous Solvents

Solubilities of cyanocobalamin were determined¹¹⁶ in various solvents at room temperature with about one minute of mixing. Results are reported using the U.S.P. definitions.¹¹⁷

Solvent	Solubility
Water	Slightly soluble
Hydrochloric acid, 0.1M	Slightly soluble
Sodium hydroxide, 0.1M	Very Slightly soluble
Acetone	Very Slightly soluble
Acetonitrile	Very Slightly soluble
Acetonitrile-water (1:1)	Slightly soluble
Chloroform	Practically insoluble
Dimethylsulfoxide	Slightly soluble
Ethanol	Slightly soluble
Ethyl ether	Slightly soluble
Hexanes	Very Slightly soluble
Methanol	Slightly soluble
Methanol-water (1:1)	Slightly soluble
n-Octanol	Practically insoluble
Propylene glycol	Practically insoluble

4.3 Partition Coefficients

Cyanocobalamin was partitioned¹¹⁸ between hexanes and distilled water and between chloroform and distilled water at 22°. After mixing, the vitamin content of both phases was determined by spectrophotometry at the 361 nm maximum (*cf.* Section 5.2, spectrophotometry). Blanks consisted of the solvent saturated with the other phase. The partition coefficient of hexanes/water was found to be 0.087 and for chloroform/water it was 0.023. At 21°, partition coefficients¹¹⁹ for phenol/water (pH 6.2) was 0.055, and at 22°, for butanol/water (pH 6.0) it was 0.045, using the maximum at 361 nm.

Cyanocobalamin and hydroxocobalamin were determined¹²⁰ on the basis of partitioning in benzyl alcohol and water (1:1), again using spectrophotometry at 361 nm. The partition coefficient in this system was 1.2. Other cobalamins can be separated by counter-current distribution using the system.¹²¹

4.4 Ionization

Using the dependance of the proton-NMR chemical shift⁸⁸ on pH, base atom B-2 (*cf.* Section 1.2 for location of the atom) gave a pK of 3.28 ± 0.04. This value is in excellent agreement with the previously-reported value¹²² of 3.3. pK values for cobalamins and cobinamides have been

discussed.¹²³ The limiting conductance of the cyanocobalamin ion is 33 mhos.¹²⁴

4.5 Hydrodynamic Properties

The partial specific volume¹²⁵ of cyanocobalamin at 25° was found to be 0.662 and the diffusion constant was $2.9 \times 10^{-6} \text{ cm}^2 \text{ sec.}^{-1}$. Using these values, the calculated molecular weight is 1380, in excellent agreement, considering the errors of these techniques, with the theoretical M.W. of 1355 daltons. Using ultracentrifugation,¹²⁶ the diffusion constant was found to be $2.7 \times 10^{-6} \text{ cm}^2 \text{ sec.}^{-1}$ and the sedimentation coefficient, $S_{20,w}$ was $0.54 \times 10^{-13} \text{ sec.}$ at 0.01%. At a concentration of 1%, $S_{20,w} = 0.6 \times 10^{-13} \text{ sec.}$

5. Methods of Analysis

5.1 Compositional Analysis

5.11 Elemental Analysis

The elemental analysis¹²⁷ of cyanocobalamin is as follows:

<u>Element</u>	<u>Found</u>	<u>Theoretical</u>
Carbon	52.40	52.51
Hydrogen	6.83	6.83
Nitrogen	13.62	13.66
Phosphorus	2.1	2.28

5.12 Water Content

Based on an apparent molecular weight of 1443.95-1355.42 (actual) = 88.53 daltons, 4.9 molecules of water or 6.1% moisture were present in a commercial preparation. Vapor phase chromatography¹²⁸ was used to analyze for water in a commercial preparation of cyanocobalamin. The vitamin was dissolved in pyridine and, after retention on a precolumn, water content of 5.7% was determined by comparison with external standards.

5.13 Emission Analysis

Emission spectrochemical analysis was performed.¹²⁹ A commercial preparation of cyanocobalamin was found to contain 3.9% cobalt, which, after correction for water, is 4.35% (4.39%, theoretical content). Phosphorus content is 2.3%, in excellent agreement with the results found by elemental analysis. Metallic impurities in $\mu\text{g/g}$ were: zinc, 38; iron, <5; aluminum, <5; magnesium, <5, and calcium, 4. Emission spectroscopy has been used to determine the purity of vitamin B₁₂ at the 3435.5Å cobalt analytical line.¹³⁰

5.14 Atomic Absorption

More recently, atomic absorption methods have been used to determine cyanocobalamin in bulk¹³¹, in dosage forms¹³²⁻¹³⁵, and in dry feeds.¹³⁶ Inorganic cobalt is usually the standard.¹³⁶ Interferences by phosphate and other ions are minimized by nebulising the cobalt into the flame in a solution of 8-hydroxyquinoline.¹³⁷ The determination of cobalt in various forms in blood has been reviewed¹³⁸ and a standard method given. Blood and serum are oxidized with a mixture of nitric, perchloric and sulfuric acids. Cobalt is extracted from the aqueous ash using 1-nitroso-2-naphthol in chloroform prior to atomic absorption analysis. The limit of detection is 10^{-11} g. Recovery¹³⁹ of ⁵⁷Co is 99.8±0.1%. Prior to the assay of cyanocobalamin in tissues, enzymatic hydrolysis (proteolysis) is often useful.

5.15 Titration

An iodometric titration for cobalt in ashed cyanocobalamin is claimed to have an error of ±0.5%.¹⁴⁰ A perchloric acid titration of cyanocobalamin in glacial acetic acid indicated at least six weakly basic groups.¹⁴¹

5.2 Identity, Spectrophotometric and Colorimetric Methods

5.21 Identity Tests

Compendial methods¹⁴² involve (1) fusion of cyanocobalamin with potassium pyrosulfate and reaction with nitroso R salt solution, (2) digestion and acetous ammonium cyanate addition and (3) reaction with hypophosphorous acid, distillation and the addition of ferrous ammonium sulfate solution, sodium fluoride and acid. All of these methods yield strong colors.¹⁴³

A spectrophotometric identification test¹⁴⁴ is based on the ratio of absorbance at 361 nm to 278 nm being between 1.70 and 1.90, and the ratio A_{361}/A_{550} being between 3.15 and 3.40. The maxima should be ± 1 nm at 361 and 278 nm, and ± 2 nm at 550 nm.

5.22 Spectrophotometric Methods

Aqueous solutions exhibit absorption maxima¹⁴⁴ in the ultraviolet and visible regions (*cf.* Section 3.5). Using traditional nomenclature, at 278 nm ± 1 nm, $E(1\%, 1\text{ cm}) = 115$, at 361 nm ± 1 nm, $E(1\%, 1\text{ cm}) = 207$, and at 548 nm ± 3 nm, $E(1\%, 1\text{ cm}) = 63$. A compendial assay¹⁴⁵ is based on the comparison of the sample in water with the absorbance of an authentic standard at the peak maximum of 361 nm. Subdued light should be used since aqueous cyanocobalamin is converted by light to hydroxocobalamin, which has a lower absorptivity at 361 nm.¹⁴⁴ Alternatively, the isosbestic for the two compounds or absolute ethanol can be used. Spectrophotometric determinations¹⁴⁶ must be compensated for variable hydration of various lots of cyanocobalamin.

Vitamin B₁₂ has been determined in the presence of light-scattering components¹⁴⁷, hydroxocobalamin^{148,149}, related corrinoids¹⁵⁰⁻¹⁵⁵, other vitamins^{156,157,158}, and biological constituents.¹⁵⁹ Cyanocobalamin has also been extracted with 0.025% sodium nitrite prior to spectroscopy¹⁶⁰, or with benzyl alcohol prior to determination as the dicyanide.¹⁶¹ A differential assay in

acidic and basic solution was developed.¹⁶²

The infrared absorption band of the cyano-group at 2137 cm^{-1} has been used to quantitate cyanocobalamin. Using a KBr pellet¹⁶³, the error is $\pm 5\%$. In benzyl alcohol¹⁶⁴ the error is $\pm 2\%$.

The fluorescence of the 5,6-dimethylbenzimidazole moiety, after acid hydrolysis and extraction into organic solvents, has been used to determine the cyanocobalamin content of pure solutions.^{165,166,167} Excitation at 275 nm and emission at 305 nm has been used¹⁶⁸ to quantitate the vitamin at concentrations of $0.1\text{ }\mu\text{g/ml}$.

5.23 Analysis by Chemical Reaction of a Functional Group

5.231 Cobalt

Tabulated below are selected methods for quantitating cyanocobalamin based on cobalt content.

<u>Reference</u>	<u>Principle and Comments</u>
169	Colorimetry, linearity 2 to 20 $\mu\text{g Co/ml}$
170	<i>N</i> -Bromosuccinimide, titration, 99.2% Accuracy
171	Colorimetry, selective
172	EDTA complex, 412 and 418 nm maxima
173	Colorimetry, selective
174	Complex, gas-liquid chromatography
175	Complex, spectrophotometry
176	Chemiluminescence, detection to $2 \times 10^{-9}\text{ M}$
177	Chemiluminescence, $0.07\text{ }\mu\text{g Co/mL}$
178	Complex, colorimetry, many interferences

- 179 Complex, linearity 0.23-4.5 $\mu\text{g Co/mL}$
180 Complex, poor sensitivity
181 Colorimetry nitroso-R salt, 100 to
182 { 600 $\mu\text{g B}_{12}$ quantitated
183 Colorimetry, kinetic quantitation
184,185 Ozonization, complex with EDTA
186 Ozonization, 13% error
187 Complex, 5% error
188 Complex, linearity 1-15 $\mu\text{g/mL}$
189 Complex, linear response at 570 nm

5.232 Cyanide

Cyanocobalamin is 1.92% cyanide.
Summarized below are selected assays based on
cyanide content.

<u>Reference</u>	<u>Principle and Comments</u>
190	Complex, relative standard deviation of 2%
191	Irradiation, complex, and colorimetry
192,193	Automated, recovery of 94-103%
194	Complex, error of 4%
195	Complex with 2 agents, Recovery of $\sim 100\%$
196	Microdiffusion and colorimetry
197	Complex, spot test

5.233 Other Functional Groups

The Kuhn-Roth method for oxidizing
C-methyl groups with chromic acid in sulfuric
acid, reducing excess reagent with hydrazine

and adjusting the pH to liberate acetic acid has been used¹⁹⁸ to analyze for cyanocobalamin. The approximately 50 μg of acetic acid formed was determined by chromatography rather than the usual titration.

Vitamin B₁₂ has been quantitated based on the phosphate content after decomposition of the vitamin as phosphomolybdate.¹⁹⁹ The molybdate can be determined either colorimetrically as the thiocyanate (sensitivity: 0.01 μg P/mL or 0.43 μg cyanocobalamin/mL) or polarographically in nitric acid-ammonium nitrate solution (sensitivity 0.01-0.02 μg P/mL).

The hydrolysis of vitamin B₁₂ in hydrochloric acid gives 5,6-dimethylbenzimidazole. This compound has been determined colorimetrically in a multistep reaction via 4,5-dimethyldibenzoyl-*o*-phenylenediamine (II) with acetylacetone.²⁰⁰ Alternatively, II can be reacted with alloxan to form 6,7-dimethylalloxazine, which fluoresces. Reproducibility of both methods is approximately $\pm 2\%$.

5.3 Chromatographic and Other Separation Analyses

5.31 High-Pressure Liquid Chromatography (HPLC)

To this author, the preferred methods are chromatographic since retention time usually depends on the interactions of the molecule, via weak bonding forces, with the mobile and stationary phases.

HPLC was used by Woodward and coworkers in 1971 to purify intermediates in the synthesis of cyanocobalamin and to determine the purity of synthetic vitamin B₁₂ using normal phase chromatography²⁰¹ (*cf.* Introduction). The preparative column was 240 x 2.3 cm and packed with 37-80 μ silica gel particles. The mobile phase was hexane-isopropanol-methanol (5:2:1) flowing at 34 mL/min. The size of the injected sample was 5 g. Smaller samples were analyzed on a column 180 by 0.2 cm, inner diameter, containing 37 μ silica. Better

resolution of the various components can be achieved presently using 5 or 10 μ columns. Other HPLC procedures are summarized below.

<u>Reference</u>	<u>Analyte or Matrix</u>	<u>Parameters and Comments</u>
202	Bulk	Octadecylsilane (ODS) column, aqueous 12% acetonitrile (0.7 mL/min.), UV
203	Analogues	5 μ ODS, 37% methanol-0.04 M tartarate-disodium hydrogen phosphate buffer, pH 3.0 (0.83 mL/min.), 254 nm
204	Cobalamins	ODS; gradient, 0.05 M sodium acetate (pH 4.0) or 0.05 M sodium dihydrogen phosphate and methanol (1.8 mL/min) 254 nm.
205	Cobalamins	Octylsilane, 30% acetonitrile-70% water (2 mL/min) or gradient; 0.083 M phosphoric acid (pH 3.3) with triethanolamine and acetonitrile from 10 to 25% in 10 min.
206	Analogues	Ethylsilane, 40% (1% acetic acid in water)-60% methanol (1 mL/min), 360 nm.
206	Analogues	ODS, 90% methanol-0.2% aqueous ammonia (2.6 mL/min).
207	Analogues	Cyanocobalamin is not resolved from methyl cobalamin, 360 nm.
208	Vitamins	ODS, gradient; 0.001 to 0.5 M potassium dihydrogen phosphate solution.

209	B vitamins	ODS, 20% (1% citrate in water)-80% methanol (pH 7.3).
209	B vitamins	Amino column, 20% water-80% methanol.
210	B vitamins	Amino column, 95% methanol-5% water (1 mL/min.), 266 nm.
211	Vitamins	Amino; gradient, 0.005 <i>M</i> potassium dihydrogen phosphate-86.5 to 63% acetonitrile in 8.7 minutes.
211	Folic acid and B ₁₂	Octylsilane column, 0.005 <i>M</i> ammonium acetate-acetonitrile (85:15).
212	B vitamins	Cation exchange column, 2 <i>M</i> ammonium chloride (pH 4), 1 mL/min.
213	Vitamins	ODS, 1% aqueous acetic acid-acetonitrile (90:10). 280 nm.
213	Vitamins	Extract into metabisulfite buffer, ODS column acetonitrile-0.005 <i>M</i> oxalic acid, pH 4.1 (15:85) 546 nm.
214	Vitamins and Cobalamins	Amino column at 40°, 0.005 <i>M</i> phosphate buffer (pH 4.35), acetonitrile (23-77%, 4 mL/min, 220 nm. Low error of 1.2%, Hydroxocobalamin is not resolved.
215	Sludge	Reverse-phase column (no details given - ODS assumed), 0.01 <i>M</i> potassium dihydrogen phosphate-methanol (3:1), 0.5 mL/min., 550 nm.

216	Veterinary formulation	Octylsilane; gradient, 15-45% methanol-0.05% sulfuric acid, 1.3 mL/min., 350 nm.
217	Corrins <i>in vivo</i>	ODS; 50 mM sodium dihydrogen phosphate and methanol in combination of gradient and isocratic system. Extraction is necessary. 254 nm.
218	Various	Electrochemical detector (ECD). Disadvantage is inability to detect resolved compounds in the same oxidation state as that produced by the detector. Advantage is that the more sensitive ECD may be used to analyze cyanocobalamins in tissues and body fluids.

Figure 10 shows liquid chromatograms of cyanocobalamin separated from a mixture of vitamins (A) and from folic acid (B), taken from reference 211.

The figure exemplifies the use of micro-processor controlled HPLC equipment. Sample and standard solutions, in vials, can be automatically injected into a chromatographic system using a mobile phase that can separate multiple components. The assay results can be displayed with parameters indicating the validity of the assay.

FLOW 3.00 2.99 ml/min
 C.B. 85.5 86.5 bar
 COLUMN P 100
 MAX P 400
 MIN P 0
 OVEN TEMP 65 65 °C
 EXT. SGNL
 CHY SPD 1.00
 RTTN 2T 7
 CERO 10.0
 SLP SENS 80.00
 APEN FEJ 100
 OFTN 4

Sample size: 10 µl

Sample size: 10 µl

Column:
 25 cm x 4.6 mm i.d.
 LiChrosorb

NH₂, 10 µm

Mobile phase:

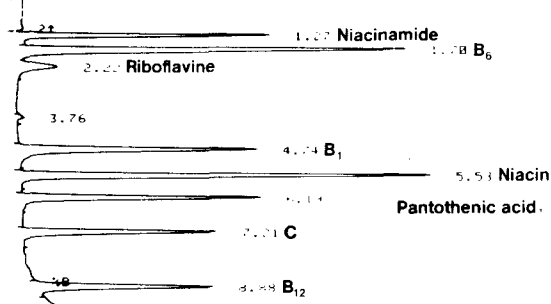
A 0.005 M KH₂ PO₄

B acetonitrile.

time.

1.05 SLP SENS 8.50
 8.70 10 85.0
 4.50 REPORT
 12.00 10 85.5
 15.00 STOP

IN START



10300

ESTD	PUN #				
PT		PT	AREA	CAL #	AMT
1.27		1.26	56350	2	535.184 ng/10 µl
1.70		1.70	102100	3	518.682
2.22		2.23	20000	4	454.960
4.74		4.75	71300	5	1085.78
5.53		5.52	107600	6	437.386
5.19		5.20	63300	7	1937.32
7.21		7.21	52700	1	5291.20
8.88		8.88	44620	8	512.816

F: 1.0000 E+ 0

A

Figure 10. High-Pressure Chromatography of Cyanocobalamin.
 A. Gradient analysis of water-soluble vitamins
 (standard solution).

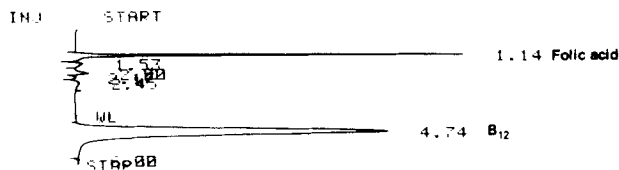
FLOW 2.00 1.98
 INB 15.0 14.5
 COLUMN P 54
 MAX P 400
 MIN P 0
 S-TEMP A 40 41
 S-TEMP B 25 25
 OVEN TEMP 40 40
 VM SGNL
 WAVL S:R 278 : 600
 CHT SPD 0.50
 ZERO 10.0
 ATTN 2† 3
 AREA PEJ 3000
 SLP SENS 0.20

Sample size:
 10 µl
 Column:
 25 cm x 4.6 mm i.d.
 LiChrosorb® RP-8,
 10 µm

Mobile phase:
 0.005 M ammonium
 acetate buffer/
 acetonitrile (85/15)

a) Standard Solution
 b) Solution of Tablet

4.00 WAVL S:R 212 : 0



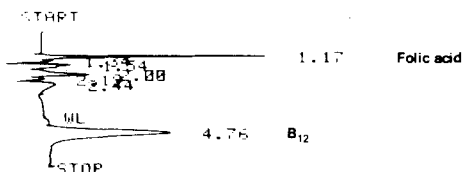
HP 1080 B sample: test

BTL: 9
ID: 17-7-78-A300

RT	AREA	AREA %
1.14	94300	18.616
1.53	7250	1.400
2.00	10740	2.169
2.45	5890	1.130
4.74	345000	76.115

DF: 1.0000 E+ 0

INJ sample: tablet



HP 1080 B

BTL: 15
ID: 17-7-78-A300

RT	AREA	AREA %
1.17	13050	18.661
1.34	3320	4.241
1.54	8450	10.788
2.00	11300	14.452
2.44	6704	8.559
4.76	35400	45.298

DF: 1.0000 E+ 0

B

Figure 10. B. Isocratic analysis of folic acid and vitamine B₁₂. Courtesy of Hewlett-Packard Corp.

5.32 Thin-Layer Chromatography (TLC)

TLC can be used to separate and estimate or quantitate cyanocobalamin in various materials. The precision and accuracy of the method depend on the concentration, amount of "bound" vitamin B₁₂, method of detection, and skill of the analyst to spot or streak, develop and assay the regions on the plate. For quantitation, scraping the plate and eluting the vitamin, followed by detection yield the best results. Table 1 summarizes TLC methods for cyanocobalamin. Toluene should be substituted for benzene for health reasons.

5.33 Paper Chromatography

Paper chromatography is presently used less frequently because of the superior resolving power, and ease of HPLC and TLC. In the past, it was used to determine the purity of cyanocobalamin²⁵⁶⁻²⁶¹, to separate cyanocobalamin from related corrins²⁶²⁻²⁶⁸, and to separate vitamin B₁₂ from other vitamins²⁶⁹ and from such other substances as cobalt²⁷⁰⁻²⁷¹, methionine²⁷², nucleotides²⁷³ and penicillin, using in this instance, a single thread of cotton.²⁷⁴ The chromatographic system must be protected from light to prevent cobalt-cyanide cleavage.

5.34 Ion-Exchange Chromatography

Ion-exchange chromatography, generally using a column open at the top and filled with a resin, has been used to purify or determine the purity of cyanocobalamin²⁷⁵⁻²⁸⁶, to separate cyanocobalamin from other corrins²⁸⁷⁻²⁹⁴, and to separate vitamin B₁₂ from other vitamins²⁹⁵⁻³⁰⁶, and other constituents in liver^{307,308,309}, serum³¹⁰, neomycin³¹¹, feed mixtures³¹², orange juice³¹³, nucleotides^{314,315} and syrups^{316,317}. HPLC and TLC are generally faster and more convenient, precise and accurate.

5.35 Size-Exclusion Chromatography

Exclusion gels reject larger molecules and compounds from their interiors, while interacting and retarding the elution of smaller species.

Table 1: Thin-Layer Chromatography of Cyanocobalamin

<u>Support</u>	<u>Mobile Phase</u>	<u>Detection</u>	<u>Reference</u>
<i>Cyanocobalamin</i>			
Silica gel	Glacial acetic acid-acetone-methanol -benzene (5:5:20:70), or water	Visible, radioactive	219
Silica gel with acetate	Methanol-water (19:1)	360 nm	220
Dextran gels and polyethyl- ene micro particles	-	-	221
Silica gel, cellulose	Methanol-water (19:1)	Visible, <i>o</i> -toluidine	222
Sodium silicate on Aluminum foil	Acetic acid-methanol (3:1)	-	223

Table 1 - TLC, continued

	<u>Support</u>	<u>Mobile Phase</u>	<u>Detection</u>	<u>Reference</u>
	<i>Corrins</i>			
	Silica gel	Butanol-acetic acid-0.066 <i>M</i> KH ₂ PO ₄ -methanol (4:2:4:1)	361 nm	224
	Silica gel	0.4% Pyridine, 3% phenol, 0.01% NaCN and 10% acetic acid, thymol saturated	Visible	225
230	Alumina G	Acetic acid-H ₂ O-methanol-CHCl ₃ - butanol (2:9:10:20:50)	Bioauto- graphy	226
	Silica gel	Anhyd. acetic acid-H ₂ O-methanol- CHCl ₃ -butanol (9:11:5:10:25)	Bioauto- graphy	226
	Silica gel or aluminum oxide	Methanol-2% aqueous KCN (19:1)	Visible	227
	Cellulose	2-BuOH-0.1 <i>M</i> acetate buffer, pH 3.5-methanol (4:12:1), lower layer	-	228
	Alumina	2-BuOH-2-propanol-water (1:1:1)	Visible	229,230
	Cellulose	<i>Sec</i> -butyl alcohol-water (19:8)	Visual	231

Table 1 - TLC, continued

	<u>Support</u>	<u>Mobile Phase</u>	<u>Detection</u>	<u>Reference</u>
	Cellulose	<i>Sec</i> -Butyl alcohol-acetic acid-water (100:1:50)	Visual	231
	Cellulose	Butanol-acetic acid-water (9:1:15) upper layer	Visual	231
	Alumina	Isobutyl alcohol-isopropyl alcohol-water (1-1.5:1:1)	360 nm	232
231	Silica gel	Butanol-methanol-water (30:30:15)	-	233
	Silica gel or Cellulose	<i>Sec</i> -Butanol-NH ₃ -water (190:5:55)	Visual Bioautography	234
	Silica gel	Ethanol-water (70:30)	-	235
	Silica gel	Butanol-acetone-water (48:48:4)	361,545 nm	236
	Silica gel	2-Butanol-2-propanol-water-conc. NH ₄ OH (50:50:50:1)	Radioactivity	237

Table 1 - TLC, continued

<u>Support</u>	<u>Mobile Phase</u>	<u>Detection</u>	<u>Reference</u>
Silica gel, silanized	Acetic acid-methanol-water (1:60:140)	Radioactivity	237
Microcrystalline cellulose	<i>n</i> -Butanol-ethanol-water (10:3:7) plus 0.5% NH ₄ OH	Visual	238
<i>Separation from other B vitamins and vitamin C</i>			
Silica gel	Acetone-isopropyl alcohol- 12% aqueous ammonia	Colorimetry	239
Cellulose	Propanol-water (6:4) or propanol-ethyl acetate- NH ₄ OH (5:3:2)	-	240
Silica gel	Methanol-water (95:5)	254 nm	241
Silica gel	Butanol-acetic acid-water (4:1:5)	-	242
Silica gel	Propanol-ethyl acetate-water (2:1:4)	-	242

Table 1 - TLC, continued

<u>Support</u>	<u>Mobile Phase</u>	<u>Detection</u>	<u>Reference</u>
Silica gel	Butanol-water-acetic acid (120:60:24)	Visual	243
Silica gel	<i>Sec</i> -Butanol-ethanol-water- 32% NH ₃ (50:30:19:1)	Visual	243
Silica gel	Ethanol-water (2:1)	Colorimetry	244
<i>Cyanocobalamin in Multivitamin Formulations</i>			
Alumina	Acetate-acetic acid-methanol- benzene (1:1:4:14)	<i>o</i> -toluidine	245
Silica gel	Water-96% ethanol-2 <i>M</i> HCl (45:48:0.2)	Visual	246
Silica gel	Benzene-petroleum ether-acetic acid (35:65:1)	Multiple colorimetric reactions	247
Silica gel	Acetone-acetic acid-benzene- methanol (1:1:14:4)	Visible	248
Silica gel	Water	Cl- <i>o</i> -toluidine	249

Table 1 - TLC, continued

<u>Support</u>	<u>Mobile Phase</u>	<u>Detection</u>	<u>Reference</u>
Silica gel	Benzene-light petroleum-acetic acid (35:65:1)	5% ethanolic molybdophosphoric acid	250
Silica gel	6 solvent systems	-	251
<i>B₁₂ in liver</i>			
Silica gel	50% aqueous ethanol	0.1% KCN, acetic acid, 550, 525 nm	252
<i>B₁₂ in plasma</i>			
Cellulose and silica gel (3:1)	<i>Sec</i> -Butanol-ammonia water (75:2:25)	Bioautography	253 254
Silica gel	2-Butanol-2-propanol-water-ammonia (30:45:25:2)	Bioautography	255

Sephadex resins have been used to separate cyanocobalamin from other corrins^{318,319}, from sea water³²⁰, urine, plasma and hemodialysis fluid³²¹, plasma³²², gastric juice³²³ and liver^{324,325}. As with all chromatographic techniques, interference by other substances is possible; for example, sulfitecobalamin interferes³²⁶ with cyanocobalamin in an exclusion separation utilizing Sephadex C-25. Vitamin B₁₂ has been used as a marker to determine the elution volume corresponding to its molecular weight.³²⁷

5.36 Other Methods of Separation, Including Adsorption, Ultrafiltration and Partitioning

Adsorption on charcoal has been used to purify cyanocobalamin from sewerage³²⁸, to concentrate it from urine³²⁹, and, when coated on a molecular sieve, to separate vitamin B₁₂ from serum³³⁰ (*cf.* Radioassays).

Membrane ultrafiltration^{331,332,333} and dialysis tubing^{334,335} have been used to separate free from bound forms of cyanocobalamin.

Cyanocobalamin has been separated from related corrins by simple (one-pass) partitioning.^{336,337} Multiple partitioning and counter-current distribution has been used to separate vitamin B₁₂ from rat erythrocytes³³⁸, other corrins^{120,121}, and from bacterial cells.³³⁹ Electroflotation³⁴⁰ has been used to purify cyanocobalamin. Benzoquinone and naphthoquinone, which combine with nitrogenous compounds, have been used to complex³⁴¹ vitamin B₁₂. The complex can be removed by carbon and the quinone regenerated.

5.4 Electrochemical Analysis

5.41 Electrophoresis

Paper electrophoresis was used to separate cyanocobalamin from related compounds³⁴² in 2M acetic acid for 16 hours at 270 V, using bioautography to detect activity. The duration was shortened to 1.5 hours²⁶⁰ by using barbital buffers, pH 8.0 and 9.0, or to 3 hours using phosphate

buffer at pH 7. Various corrins³⁴⁴ in sheep rumen were released from their bound forms by hydrolysis and then separated from one another by high-voltage paper electrophoresis in dilute acetic acid.³⁴⁵ Sodium acetate, sodium borate and ammonium chloride electrolyte were used to separate and identify 12 water-soluble vitamins.³⁴⁶ An isoelectric focusing³⁴⁷ separation has been described. Unfortunately, complexes may have formed between the vitamin and the ampholyte. The isoelectric point of cyanocobalamin is 1.5, as found by electrophoresis.³⁴⁸

5.42 Polarography and Related Techniques

Redox processes for cyanocobalamin have been investigated using polarography³⁴⁹⁻³⁶¹, coulometry^{362,363,364}, cyclic voltammetry³⁶⁵⁻³⁶⁸ and chronopotentiometry.³⁶⁹ Oscillopolarography has been used to identify vitamin B₁₂ in aqueous solution.³⁷⁰ Cyanocob(III)alamin is reduced to B_{12r}, cob(II)alamin, with a half-wave potential of -0.63V. Further reduction gives B_{12s}, cob(I)alamin (*cf.* section 1.6). The B_{12r}-B_{12s} couple depends on the kinetics of the base-off/base-on reaction³⁷¹, as studied by cyclic voltammetry at various pH's. Vitamin B₁₂ may be regenerated by oxidation. Assays for cyanocobalamin and related compounds have been developed using alternating current polarography in dimethylformamide³⁷² at concentrations of 0.1 to 0.5 mM. *Cf.* section 5.31 for a description of an HPLC separation using an electrochemical detector.

5.43 Determination by an Ion-Selective (Cyano) Electrode

Bulk cyanocobalamin, B₁₂ in a multivitamin capsule and B₁₂ in a liver hydrolysate can be reduced with either ascorbic acid, tin(II) chloride in hydrochloric acid, or calcium hypophosphite to quantitatively release hydrocyanic acid. Alternatively, hydrocyanic acid can be released photochemically, using 500 watt lamps. The hydrocyanic acid is detected³⁷³ at microgram concentrations using a cyanide-selective membrane electrode, with an error of $\pm 5\%$. Cyanide can also be determined by titration with silver nitrate at the electrode.

In a simpler procedure³⁷⁴, cyanocobalamin is irradiated at pH 3-4, and the released cyanide is quantitated with a cyanide-selective electrode at a pH of 12-13. Recoveries are 98-101% in the presence of other corrins, indicating specificity of the method. Similarly, vitamin B₁₂ can be determined in the presence of cobinamide and hydroxocobalamin.³⁷⁵

5.5 Radioassays

5.51 Introduction and Example Assays

Radioassays are rapid convenient, cheap and generally unaffected by the presence of antibiotics, antimetabolites and tranquilizers. Cyanocobalamin can be labeled with ⁵⁶Co, ⁵⁷Co, ⁵⁸Co and ⁶⁰Co by adding labeled cobalt chloride to the fermentation medium of microorganisms producing vitamin B₁₂. Counting efficiencies are good to very good for ⁵⁶Co, ⁵⁸Co and ⁶⁰Co. For ⁵⁷Co, the counting efficiency varies with the type of counter used, but is very good for the scintillation type. The total liver absorbed doses³⁷⁶, in rad/ μ Curies are: ⁵⁶Co, 1.2; ⁵⁷Co, 0.3; ⁵⁸Co, 0.6 and ⁶⁰Co, 8.3, indicating one reason for the popularity of ⁵⁷Co and the paucity of uses, *in vivo*, for ⁶⁰Co.

The classic and most reliable radioassay is the Schilling test^{377,378,379}, which is used with borderline cases of B₁₂ deficiency; *i.e.* 0.2 μ g B₁₂/100 ml plasma.³⁸⁰ The Schilling test is an assay *in vivo* which measures the extent to which cyanocobalamin can be absorbed through the terminal ileum by examining the absorption of a small quantity of radiolabeled vitamin B₁₂ competing with a large amount of nonradioactive cyanocobalamin for receptor. It is more a diagnostic technique measuring the ability of the body to absorb B₁₂ than an assay for cyanocobalamin.

The determination of the tissue³⁸¹ or body fluid content of cyanocobalamin requires the complete separation of B₁₂ from various binders, usually by hydrolysis of homogenized tissue in 0.5 M HCl for 15 min. at 100° or by boiling in acetate-cyanide buffer. In this binding type of assay,

the cyanide converts other forms of B₁₂ into cyanocobalamin. A known quantity of labeled vitamin B₁₂ is added, the homogenate shaken or boiled, and a binder or "carrier" capable of binding 2/3 to 3/4 of the labeled cyanocobalamin is added. From the pool of mixed labeled and unlabeled vitamin B₁₂, the carrier will remove a fraction equal to the binding capacity of the factor.³⁸² Bound cyanocobalamin is separated from free B₁₂ by various methods (*cf.* Section 5.53, Technical Aspects), including chromatography and coated charcoal. The charcoal is coated with a high molecular weight compound like hemoglobin, dextran or albumin. The coating insures that only small molecules will be adsorbed. (Hemoglobin-coated charcoal is prepared by suspending 5g of a pharmaceutical grade of charcoal in 100 ml of distilled water. Human red blood cells are washed with 0.9% sodium chloride solution, then hemolyzed with twice the volume of distilled water, and then 0.5 volume of toluene is added. After shaking and centrifuging, the bottom layer is collected and filtered. Hemoglobin solution equivalent to 0.25 g in 100 mL is added to the charcoal suspension and shaken. The capacity of the binder is determined and portions frozen.) The binder, a known amount of labeled cyanocobalamin and the unknown quantity of labeled B₁₂, are allowed to react at room temperature for 30 min. After adding 2 mL of coated charcoal suspension, mixing, and centrifugation (or other separation, *cf.* Technical Aspects section), the supernatant radioactivity is determined.

$$\frac{\text{pg B}_{12}}{\text{per ml}} = \frac{1}{n} \times \text{pg } ^{\ell}\text{Co-B}_{12} \left(\frac{B}{B'} - 1 \right)$$

Where n = mL of serum assayed, B = net counts per minute of binder-concentrate control tube and B' = net counts per minute of the tube with unknown serum.

Simplified methods involve commercial kits.³⁸³ These convenient tests have reduced the reagent cost per assay of cyanocobalamin in body fluids to less than a dollar and the time to less than an hour. The general basis requires labeled cyanocobalamin to be mixed with the substrate, usually

serum. Once labelled and unlabeled vitamin B₁₂ have equilibrated, an equal fraction of each form should be bound to a matrix selective for cyanocobalamin. (At least three distinct types of protein have an extremely high affinity for cyanocobalamin.³⁸⁴ Intrinsic factor (*cf.* Introduction,) a protein secreted by the stomach, is extremely specific ($K = 6 \times 10^9 M^{-1}$). Transcobalamin II, a polypeptide in plasma, binds cyanocobalamin ($K \sim 3 \times 10^{11} M^{-1}$) and some analogues. The glycoprotein, R. protein, binds analogues³⁸⁵ as well as vitamin B₁₂, and cannot be used in radiodilution assays because of the resulting erroneous values. Some analogues are inert, some may have cyanocobalamin activity and some may inhibit vitamin B₁₂.) After separation, the ratio of labeled to unlabeled cyanocobalamin is determined by some radioactive counting technique. From the initial concentration of labeled vitamin B₁₂, the content of unknown cyanocobalamin is calculated. Unfortunately, the kit assay fails in 10% to 20% of the patients tested³⁷⁷. The problem is that usually these patients either have very low B₁₂ levels or the B₁₂ is bound and is not released, requiring further testing using either microbiological assays or the Schilling radioassay, as previously mentioned.

A newer method is the competitive protein binding (CPB or radioimmunoassay) radioassay. It involves preparing a stable derivative of cyanocobalamin^{386,387,388} suitable for eliciting a high-titer antiserum specific for vitamin B₁₂. Bound to a surface³⁸⁹ to increase the surface area and facilitate separation of free from bound cyanocobalamin, 1 ml of preparation can coat 20,000 polypropylene tubes. Sensitivity is 1.5 picograms^{390,388} of cyanocobalamin per tube, with an interassay precision of 15% to 20%.

Other reviews of radioassay techniques are available³⁹¹⁻³⁹⁶.

5.52 Radioassays for Cyanocobalamin in Various Materials

A selected list of radioassays in various matrices is tabulated below.

<u>Matrix</u>	<u>Reference</u>	<u>Comment</u>
Blood	397	Sephadex-intrinsic factor binders
Blood	398	Receptor-coated
Cells	399	Red blood cells
Cerebrospinal fluid	400	High sensitivity
Feces	401	Double-tracer technique
Feces	402	Also whole-body
Feces	403	Also uses ^{51}Cr
Feeds	404	20 Assays/hr.
Food	405	Rigorous extractions
Food	406	$^{14}\text{CO}_2$ from B_{12}
Food	407	Kit
Liver	408	Recovery of $0.5 \mu\text{g} \pm 5\%$
Sea Water	409	$\text{Na}_2^{14}\text{CO}_3$ uptake in diatoms
Serum	410	{ Kits, plasma values less than serum
Serum	411	
Serum	412	Plasma = serum values
Serum	413	584 Normal humans
Serum	414	Microbiological $^{14}\text{CO}_2$
Serum	415	Titration
Serum	416	Parameters examined
Tissue	417	Spleen, brain & kidney
Urine	418	{ Test renal function and plasma binding
Urine	419	
Urine	420	Also feces and liver
Vitamins	421	Sensitive and specific
Vitamins	422	$^{60}\text{Co-B}_{12}$ is tracer
Total body	423	And liver
Total body	424	Used 2 counters
Total body	425	Discusses body compartments

5.53 Technical Aspects

The most crucial parameter of most radioassays using labeled cyanocobalamin to quantitate unknown vitamin B₁₂ is the agent used to bind⁴²⁶ cyanocobalamin. The National Committee for Clinical Laboratory Standards recently announced proposals based on recommendations by the Clinical Chemistry and Hematology advisory panels of the United States Food and Drug Administration (February, 1979). Manufacturers of B₁₂ kits must substantiate purity; for example, tests should be developed for such non-specific binders as R protein (that combine with analogues of cyanocobalamin and mask B₁₂ deficiencies and thus give falsely normal results).⁴²⁷ (The metabolic role of these analogues is unknown at this time, and may have physiological significance. In the future, these may have to be quantitated.) Intrinsic factor must be assayed for purity by such methods as insensitivity to cobinamide⁴²⁸ up to concentrations of 10 ng/ml serum, inhibition by anti-intrinsic factor blocking antibody or the intrinsic factor-cyanocobalamin complex should be greater than 95% precipitated by specific intrinsic factor precipitating antibody.

The use of intrinsic factor^{427,429} as binding agent was discussed in the introductory radioassay section. Intrinsic factor has been immobilized⁴³⁰ on dextran^{431,432}, polysaccharides⁴³³ and Sephadex⁴³⁴, and conjugated to fluorescein.⁴³⁵ Some other binding agents are egg yolk⁴³⁶, magnetic particles containing fish serum⁴³⁷, saliva⁴³⁸, chronic myelogenous leukemia serum⁴³⁹, chicken serum⁴⁴⁰, human serum⁴⁴¹, oyster serum⁴⁴², rabbit serum⁴²¹, trout serum⁴⁴³, chicken serum on magnetized particles⁴⁴⁴, transcobalamin I⁴⁴⁵, transcobalamin on Sephadex⁴³² and a cell wall protein.⁴⁴⁶

Methods of separating "free" from "bound" cyanocobalamin include affinity chromatography⁴⁴⁷, bentonite⁴⁴⁸, charcoal⁴⁴⁹ (*cf.* Section 5.51) and charcoal coated with dextran⁴⁵⁰ or hemoglobin^{451,452}, column chromatography⁴⁵³, dialysis⁴⁵⁴, filtration^{455,456}, diethylaminoethyl (DEAE)-cellulose^{457,458}, Sephadex⁴⁵⁹ and Sepharose.^{460,461}

Also investigated has been the effect on radioassays of anticoagulants⁴⁶², ascorbate^{463,464}, autoclaving to destroy B₁₂ binding proteins⁴⁶⁵, bile⁴⁵⁰, contaminants of other radioisotopes⁴⁶⁶ and proteins^{467,468}, cyanide⁴⁶⁹, glass⁴⁷⁰, peptides⁴⁷¹ and tissue preparation.⁴⁷²

Various radioassays have been compared with each other; for some methods there were negligible differences^{473,474,475}, however, for several other assays there were significant discrepancies.^{476,477} Combination radioassays for cyanocobalamin and folate are available.^{478,479,480} Automated and semi-automated systems have been described^{481,482}, as have various methods of calculating results.^{483,484,485} Counting techniques have been investigated.^{486,487}

5.6 Microbiological Assays

5.61 Introduction and Example Assay

The microbiological assay for cyanocobalamin is relatively simple, selective and sensitive but, depending on the organism, may vary in specificity. This variation in selectivity⁴⁸⁸ is because the organisms contain enzymes to catalyze various transformations between analogs or release "bound" vitamin B₁₂. Despite such other handicaps as the need for meticulously cleaned glassware, growth in blanks and non-linear responses, possibility of contamination, and poor response when some antibiotics and other drugs have been administered, the utility of the microbiological method makes it first choice for many applications.

The compendial turbidimetric procedure⁴⁸⁹ has been modified to an automated assay. Such an automated assay as the AutoTurb system^{490,491} limits many variables and has been successfully used for thousands of analyses. As described below, it serves both as an example of a rugged method and an aid in understanding the references that follow to related microbiological procedures. This assay measures cyanocobalamin by its effect in stimulating the rate and amount of growth of a vitamin B₁₂-requiring *Lactobacillus leichmanii*⁴⁹² in a turbidimetric assay system. It is applicable

to bulk cyanocobalamin in various forms and pharmaceutical products.

Grind or blend 1 to 10 tablets for a few seconds to a uniform powder. Autoclave one tablet weight (amount depends on potency) or 0.5 to 10 mg of bulk vitamin, or equivalent, for 5 min. at 121° in 200 ml of bisulfite buffer. Cool, dilute with bisulfite buffer to 4 ng/ml, filter and dilute 10-fold with distilled water. Fill the tubes for the automatic system with samples and medium containing amino acids, vitamins and inorganic solids, or equivalent). Sterilize in 10 ml volumes for 20 min. at 121°. Cooled, medium-filled, tubes are inoculated with one drop of working inoculum. (The master culture is *L. leichmanii* A.T.C.C. 7830.) A frozen working culture is prepared by inoculating medium with about 10% master culture and incubating it for about 16 hours at 37°. Adjust the pH of the culture to 6.7-7 with sterile 2 M sodium hydroxide and dilute it to 7.5% with sterile glycerol. Two ml portions are frozen with dry ice and acetone, and stored at -75° until needed. When needed, 20 ml of inoculum medium are added at 37° to the frozen culture, the tube is thawed with gentle agitation and incubated at 37° for 24 hours. This working culture is diluted 1 to 100 with sterile saline prior to one drop inoculations of each B₁₂ assay tube. Samples are analyzed in quadruplicate.

Standards consist of 170-175 ng of U.S.P. reference standard, or equivalent (stored over silica gel at -20°) per ml of distilled water. Working solutions are dilutions of 1:1000, 1:500, 1:330, 1:250 and 1:200 in a 9 to 1 mixture of distilled water and bisulfite buffer. Quadruplicate assays are made also of the standards.

Inoculated tubes are incubated at 37° for 18 to 36 hours, mixed and the turbidity of each rack of tubes is measured at 600 nm using an automatic reader. Values more than twice the relative standard deviation for the set are discarded, leaving three responses. If any remaining values exceed the statistical limit, all responses for that sample are discarded.

The best linear fit of the standard responses is made; such as concentration *vs.* % transmission, or log %T, or log concentration *vs.* %T. One standard data point may be eliminated to achieve correlation coefficients above -0.991. Sample potencies are calculated using this standard plot and multiplied by dilution of the sample using a computer. Errors are less than $\pm 5\%$.

Many other laboratories analyzing large numbers of samples for cyanocobalamin use related manual⁴⁹³ or automated methods.⁴⁹⁴ *Lactobacillus leichmannii* presently is the preferred organism.

5.62 Microbiological Assays for Cyanocobalamin in Various Materials

Selected representative methods for determining vitamin B₁₂ in various matrices are tabulated below.

<u>Matrix</u>	<u>Reference</u>	<u>Comment</u>
Amino acid	495	with methionine
Antibiotic	496	with dihydrostreptomycin
Antibiotic	497	with tetracycline
Bile	498	plus chromatography
Blood	499	erythrocytes content
Blood	500	in B ₁₂ deficiency
Blood	501	micro method
Blood	502	acidity
Blood	503	for free B ₁₂
Body fluids	504	using several organisms
Bulk	505	micro method
Corrins	506	plus chromatography
Corrins	507	plus chromatography
Feed	508	with preservative
Feed	509	with vitamin B ₆
Feed	510	in clover
Feed	511	multiple constituents

<u>Matrix</u>	<u>Reference</u>	<u>Comment</u>
Feed	512	plus other vitamins
Feed	513	meat products
Feed	514	comparison of methods
Food	515	sterilized
Food	516	in rice, etc.
Food	517	in fermented milk
Food	518	plus other vitamins
Food	519	obviated interferences
Food	520	in 86 heated foods
Food	521	various foods
Food	522	eggs
Food	523	milk
Intrinsic factor	524	binding to B ₁₂
	525	
Microbes	526	extract B ₁₂
Pharma- ceuticals	527	miscellaneous
	528	miscellaneous
Serum	529	normal range
Serum	530	many samples
Serum	531	> 1 pg B ₁₂
Serum	532	total B ₁₂
Serum	533	0.05-0.2 ml
Serum	534	in anemia
Serum	535	many assays
Serum	536	total B ₁₂
Serum	537	in anemia
Serum	538	proteins precipitated
Serum	539	proteins precipitated
Serum	540	proteins precipitated
Tissue	541	liver extract
Tissue	542	100 pg B ₁₂ /ml

<u>Matrix</u>	<u>Reference</u>	<u>Comment</u>
Tissue	543	liver, + cyanide
Tissue	544	liver, + cyanide
Tissue	545	various species
Urine	546	concentrated
Water	547	in ocean
Water	548	0.1-1 pg B ₁₂ /ml
Water	549	fresh water
Water	550	1 month assay
Water	551	many methods
Water	552	plus other vitamins
Vitamins	490	multivitamins + minerals
Vitamins	553	B vitamins
Vitamins	554	C non-interference

5.63 Technical aspects

Automation has been used to achieve high rates of sample assays⁵⁵⁵⁻⁵⁵⁸, in the presence of tetracycline⁵⁵⁹ and other pharmaceuticals.^{560,561} Requirements for a successful automated system have been discussed.⁵⁶² Autodilutors have been used to increase the number of samples analyzed.⁵⁶³ Data handling has been simplified by computerization.⁵⁶⁴ Computations used to evaluate the microbiological results have been discussed.^{565,566,567} The dose response curve has been studied.⁵⁶⁸⁻⁵⁷¹ Cyanocobalamin has been assayed using the method of standard addition.⁵⁷²

Also studied has been the effect on the assay of antibiotics^{573,574,575}, cleanliness⁵⁷⁶, flask size⁵⁷⁷, growth inhibitors⁵⁷⁸, inoculum size⁵⁷⁹, preservatives⁵⁸⁰, reducing sugars⁵⁸¹, salts⁵⁸² and spectrophotometer sensitivity.⁵⁸³

Use of various types of plates has been studied.^{584,585,586} Methionine interferes⁵⁸⁷, giving lower results compared to turbidimetry.

Other strains have been studied, including *Azotobacter suis*⁵⁸⁸, *Escherichia coli* (50-1500 µg)^{589,590,591}, *Euglena gracilis* (0.25-50 pg)⁵⁹²⁻⁵⁹⁸, *Ochromonas malhamensis* (50-800 pg)^{599,600} and *Poteriochromonas stipitata*⁶⁰¹, and their variants. The number in parentheses is the assay range. *Lactobacillus leichmannii* (10-200 pg) is preferred^{489,490,602,603} especially because of the short incubation times⁶⁰⁴ and its nutritional specificity.^{605,606}

Comparative studies between strains include *Tetrahymena* vs. *Ochromonas*⁶⁰⁷, *Lactobacillus* and *Bacillus coli* vs. *Ochromonas*⁶⁰⁸, and *E. coli*, *L. leichmannii* and *E. gracilis* vs. *O. malhamensis*.^{609,610}

Turbidimetric methods have been investigated.^{611,612,613} Growth has been measured using a Coulter counter.⁶¹⁴

5.7 Enzymatic Assays

Biological reactions are usually catalyzed by cyanocobalamin in the form of the coenzyme, where the cyanide group is replaced by 5'-deoxyadenosine. Cf. the introduction.

Methionine can be determined microbiologically^{615,616} or using [¹⁴C]methylmethionine^{617,618}, using purified B₁₂ methyltransferase containing 1-2.5 µmoles of cobalamin per mg of protein. Cyanocobalamin can catalyze this reaction converting homocysteine to methionine.

Carbon skeleton rearrangements catalyzed by methylmalonyl-CoA⁶¹⁹ (E.C. 5.4.99.2) and α-methyleneglutarate⁶²⁰ (E.C. 5.4.99.x) mutases can be used to assay for coenzyme B₁₂. Glutarate mutase (E.C. 5.4.99.1), however, requires a Co-5'-deoxyadenosyl derivative⁶²¹, greatly increasing the difficulty of the assay.

Ethylamine deaminase from *Clostridium* requires α(adenyl)-Co-5'-deoxyadenosylcobamide⁶²² as coenzyme to catalyze amino group migrations. Related corrins can inhibit the enzyme.

Lysine mutase (E.C. 5.4.3.3,4) requires co-enzyme B₁₂ as cofactor⁶²³, *L*-β-Lysine mutase has been assayed by determining the amino acid content of the reaction mixture by thin-layer chromatography⁶²⁴ ninhydrin reagent⁶²⁵ or a coupled enzyme reaction.⁶²⁶

Diol hydrase (E.C. 4.2,1.28) is used to assay coenzyme B₁₂. Enzyme from *Aerobacter aerogenes*, grown in the absence of vitamin B₁₂, is added to 1,2-propanediol. The amount of propionaldehyde formed⁶²⁷ is determined colorimetrically with benzothiazol-2-one hydrazine. Propionaldehyde can also be determined in nanogram quantities using 2,4-dinitrophenylhydrazine^{628,629} or, via a coupled reaction, by the alcohol dehydrogenase reaction.⁶³⁰ Purified enzyme enables as little as 0.01 pmole of coenzyme B₁₂ to be measured using colorimetry.⁶³¹

Vitamin B₁₂, coenzyme B₁₂ and related corrins were determined in picogram quantities using glycerol dehydratase (E.C. 4.2.1.30).⁶³² The product β-hydroxypropionaldehyde was determined colorimetrically⁶³³ with a 10% error at concentrations of 5.25 picomoles.

The apo-protein form of glycerol dehydratase binds strongly, to its active center, non-coenzymatic cobamides in a stoichiometric relationship. Since this complex is inactive, the amount of cyanocobalamin is proportional to the inactivation.⁶³⁴ This method has been automated for routine assays of cyano- and hydroxocobalamin.⁶³⁵

5.8 Mass Fragmentography

Coenzyme cyanocobalamin has been quantitated using mass fragmentography.⁶³⁶ This increasingly useful technique can rapidly and selectively determine low concentrations of compounds in biological materials. The major problem with this procedure is its high initial cost. Picomole quantities of 5'-deoxyadenosylcorrinoids were assayed in biological material using the deuterated compound as internal standard. Serum samples containing 2 to 20 picomole(s) of cobalamin(s) were extracted with 10% trichloroacetic acid and centrifuged. After extraction and partial purification

using an Amberlite XAD-2 ion-exchange column, the corrinoids were eluted with *t*-butanol in 0.5% acetic acid and dried. The coenzyme was split with light, reduced with lithium borohydride and then alkylated with dimethylsulfate. After desalting, and reaction with sulfuric acid and mercuric sulfate, the samples were extracted into benzene (CAUTION). Methylated cobalamins were determined by examining the intensities of typical fragments⁶³⁷ of 8,5'-cyclic-5'-deoxyadenosine(TMS)₃ and 5,5'-d₂-8,5'-cyclic-5'-deoxyadenosine(TMS)₃.

5.9 Comparison of Methods

Greatest attention has been given to comparing radioassays with microbiological methods for the diagnosis of cyanocobalamin deficiency. Although some investigators found radioassays to be superior⁶³⁸⁻⁶⁴² or equal⁶⁴³⁻⁶⁴⁸, the general consensus (*cf.* Radioassay Sections) is that the microbiological assay^{649,650} gives a clearer distinction than the radioactive method between normal subjects and patients with untreated pernicious anemia. In addition, many post-gastrectomy and folate-deficient patients yielded subnormal results for cyanocobalamin concentrations in serum by microbiological methods but normal values by radioisotopic assay. In practice, the rapid, simple and inexpensive kit radioassays are used to screen for the normal, borderline and subnormal. The borderline and questionable cases are subsequently retested for vitamin B₁₂ content using lengthier and more costly, but more exact, procedures.

A chemical method gave similar results to a radioassay.⁶⁵¹ For determining cyanocobalamin in a ternary mixture of three vitamins, a direct spectrophotometric method was found to be superior to a column chromatographic procedure⁶⁵² utilizing spectrophotometry for the final quantitation. Cyanocobalamin appeared to be retained on the column. Spectrophotometric and microbiological methods were found to give similar results.⁶⁵³

A high-pressure liquid chromatographic procedure was found to be superior to a radioisotopic, competitive intrinsic factor binding assay for

cyanocobalamin in natural water.⁶⁵⁴ Sensitivity is from 10 to 190 picograms per mL.

The enzymatic assay using glycerol dehydratase gives similar results to spectrophotometric, microbiological (*Lactobacillus leichmannii*) and cobalt-neutron activation methods.⁶⁵⁵

Many references cited elsewhere in this review give comparative data validating the procedure. To this investigator, the only assay that should be used is one that has been previously validated for the compound in its matrix, if any. Choices, if available, must be between validated methods. Decisions could be made on the basis of simplicity, convenience, precision, accuracy, time needed per assay, cost, toxicity, stability and availability of reagents. These criteria virtually eliminate such previously-used, time consuming and tedious methods as measurement of growth in weanling rats and chickens. In general, for simplicity and accuracy, high-pressure liquid chromatographic methods are preferred for bulk vitamin B₁₂ and for vitamin B₁₂ in formulations and other, easily manipulated matrices. For sensitivity, low cost and ease, radioassay kits are preferred where great accuracy is unnecessary, or for screening many samples of, for example, human serum for cyanocobalamin deficiencies. For sensitivity without lengthy extraction or concentration steps, microbiological, some radioassay and enzymatic methods are favored. Future assays will probably include mass fragmentographic and fluorescent immunological assays.

6. Stability

6.1 Inactivation

Acid⁶⁵⁶ added to cyanocobalamin causes hydrolysis of the amide groups (in the order $e >> b, d >$ acetamide groups, $c > a, g$) and the nucleotide, and the release of 1-amino-2-propanol. In dilute acid the 5,6-dimethylbenzimidazole base was found to be protonated and dissociated from the cobalt.⁶¹ This form of cyanocobalamin is called "base-off". Vigorous acid hydrolysis yields the hexa- and heptacarboxylic acids and a lactone.⁶⁵⁷

The addition of base, 0.1 M at 100° for 10 min. in air, results in the loss of biological activity⁶⁵⁸, although many physical properties remain unchanged. This inactive vitamin is called "dehydrovitamin B₁₂", cyano[8-amino- α -(5,6-dimethylbenzimidazolyl)cobamic acid- α, b, d, e, g -pentaamide- c -lactam]. With 30% sodium hydroxide at 150°, a mixture of penta- and hexacarboxylic acids and a γ -lactam were formed⁶⁵⁹ while the nucleotide was cleaved. Barium hydroxide hydrolysis at 100° frees 5 moles of ammonia.

Spectrophotometric properties of cyanocobalamin in acidic, basic and neutral solutions have been studied.⁶⁶⁰ No significant spectral changes are visible⁶⁶¹ in methanol or water for up to 24 hours, but are seen in 0.1 M hydrochloric acid and 0.1 M sodium hydroxide.

Potassium cyanide added to a red solution of cyanocobalamin gives a purple color. The nucleotide is displaced by cyanide in an S_N2 reaction. The dicyanide is unstable.⁶⁶²

Cyanocobalamin loses activity in the presence of chlortetracycline⁶⁶³, methylparaben⁶⁶⁴ and sodium phenyldimethylpyrazolonemethylaminomethane sulfonate (novalgin).⁶⁶⁵ Flavors⁶⁶⁶ induce instability.

Although crystalline cyanocobalamin is stable up to 100° for moderate periods of time⁶⁶⁷, autoclaving solutions at 120° for 20 min. or 100° for 75 min. causes loss of activity. The stability of buffered and unbuffered solutions, from pH 4.7 to 7, protected from light, was found to be similar within the experimental error of the *Lactobacillus leichmanii* 313 assay.⁶⁶⁸

As mentioned previously, cyanocobalamins are photosensitive.⁶⁶⁹ Light splits the organometallic bond giving a 5'-deoxyadenosyl radical, which was found to be capable of further reaction, and cob(II)alamin.⁶⁷⁰ The latter was stable in the absence of oxygen. With oxygen present, hydroxycobalamin is formed, which was used as the basis of an assay for the stability of cyanocobalamin in formulations.⁶⁷¹ The rate of anaerobic photolysis

is increased by quinones, thiols and some alcohols.⁶⁵⁶

Oxygen induces a loss of 88% cyanocobalamin activity in a liver preparation.⁶⁷² 3,3-Dimethyl-2,5-dioxopyrrolidine-4-propionamide was one oxidation product.⁶⁷³ Ozone has been used to liberate cobalt from cyanocobalamin preparatory to assay (*cf.* section 5.221). Permanganate yields various acids.⁶⁷⁴

Vitamin B₁₂ was claimed⁶⁷⁵ to be stable in plasma stored at -6°, 5° and 22° for 14 weeks using radioassays. Presumably, the sum of cyanocobalamin in the plasma and B₁₂ incorporated into whatever grows at room temperature is constant.

Radiation degrades cyanocobalamin in solution⁶⁷⁶ proportional to the dose, but freezing stabilizes irradiated solutions⁶⁷⁷, as determined using ⁶⁰Co-labeled cyanocobalamin.⁶⁷⁸ (This decomposition induced by radiation can be reduced by adding 0.9% benzyl alcohol.⁶⁷⁹) Frozen solutions of cyanocobalamin and sodium chloride are less stable to irradiation than control solutions lacking saline.⁶⁸⁰

Vitamin formulations have been extensively studied for stability. Thiamine inactivates cyanobalamin.^{681,682,683} Stability in solution depends on pH.^{684,685} Ascorbic acid^{686,687} destabilizes vitamin B₁₂, especially if such metals as copper, manganese and molybdenum are present^{688,689}. Two papers, published 28 years apart,^{690,691} gave data showing that the apparent loss in B₁₂ activity due to vitamin C was restored by cyanide. This observation may explain some conflicting data on the stability of cyanocobalamin with ascorbate,^{692,693} since some assay methods utilize added cyanide and some do not.

Vitamin B₁₂ was found to be unstable in the presence of thiamine and niacinamide.^{694,695} Significant losses have been found in various capsules, tablets and liquids after one year at room temperature.^{696,697} The rate of thermal degradation has been studied and extrapolated to predict shelf-life.⁶⁹⁸ Methanol vapors caused the loss of

protected cyanocobalamin in multivitamin tablets after one month of exposure⁶⁹⁹, due to the presence of ascorbic acid and niacinamide. The effect of storage on solutions of vitamin B₁₂ and other vitamins was studied.^{700,701}

The presence of reducing agents, even in trace quantities (which may be introduced in the form of excipients in the formulation), can significantly reduce potency as a result of decomposition of the reduced form of cyanocobalamin to irreversible products.⁷⁰²

6.2 Stabilization

Solutions of cyanocobalamin have been stabilized by the pyrimidine moiety of thiamine⁷⁰³, antioxidants and chelating agents⁷⁰⁴, citric acid⁷⁰⁵, cysteine⁷⁰⁶, diisopropylammonium dichloroacetate⁷⁰⁷, iron salts^{708,709,710}, galactolactate, gluconate⁷¹¹, lactate⁷¹¹, molybdate⁷¹² (which was also claimed to degrade vitamin B₁₂ in a formulation⁶⁸⁸), phosphate buffer (pH 4.6) containing 0.8% sodium chloride⁷¹³, polyhydroxy compounds like sorbitol⁷¹⁴, potassium salts⁷¹⁵, sodium salts^{716,717}, and stomach extract.⁷¹⁸ ⁶⁰Co-labeled cyanocobalamin is stabilized against radiation-induced decomposition by adding 0.9% benzyl alcohol.⁶⁷⁹

Liver extracts have been stabilized by bisulfite⁷¹⁹ and by potassium cyanide.⁷²⁰ Cyanocobalamin has been stabilized in a rectal suppository by coating with either crystalline mannitol, or sorbitol and talc.⁷²¹

Vitamin B₁₂ is currently stabilized prior to formulation in vitamin-mineral products. The best method⁷²² appears to be adsorption on Amberlite IRP-64, methacrylic acid-divinylbenzene resin.⁷²³ The 0.1% of cyanocobalamin on resin was found to be unaffected by the acid pH of the stomach, but it was eluted in the mildly alkaline small intestine, where it was absorbed. After 24 months at 25°, coated multivitamin-mineral formulations retain 94% of their potency⁷²⁴, using a spectrophotometric assay. Tests *in vivo* show similar absorption of free and resin-adsorbed cyanocobala-

min, using ^{60}Co -labeled vitamin B_{12} . Serum concentrations of B_{12} and growth rates in test animals are higher using resin-adsorbed vitamin. Other vendors⁷²⁵ of stabilized cyanocobalamin currently use gelatin, mannitol or dicalcium phosphate.

7. Metabolism

Of the $0.5\text{ }\mu\text{g}$ of ^{60}Co -cyanocobalamin administered orally to normal control subjects, 0% to 42% of the radioactivity appeared in the feces.⁷²⁶ At $5.5\text{ }\mu\text{g}$, fecal excretion ranged from 12% to 71%, indicating a dependence on concentration. Patients with pernicious anemia excrete 72% to 96%⁷²⁷, *cf.* Radioassay section. Subjects with infections excrete 45% to 60%, indicating that infections interfere with absorption. Antibiotics also inhibit absorption of vitamin B_{12} .⁷²⁸ In normal subjects, little or no radioactivity appears in urine after the administration of labeled cyanocobalamin⁷²⁹ (*cf.* Radioassays).

After oral dosing of ^{60}Co -labeled cyanocobalamin, considerable radioactivity accumulated in the liver⁷³⁰ in inverse relationship to the dose. No radioactivity was found in plasma until 4 hours had elapsed. Peak radioactivity in plasma is found in the 8-12 hour period, with a slow decline in content with time.

Cyanocobalamin appears to be temporarily stored in the gastrointestinal tract.⁷³¹ The most important organ for permanent storage is the liver, where it has a biological half-life of approximately one year.⁷³² Aquo-, hydroxo-, methyl-, cyanocobalamin and 5'-deoxyadenosyl cobalamin (coenzyme B_{12}) appear to be interconvertible *in vivo*. Oxidation and ring-opening leads to the formation and excretion of degradation products similar to the bile pigments.⁷³³

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EMETINE HYDROCHLORIDE

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1. Introduction - History

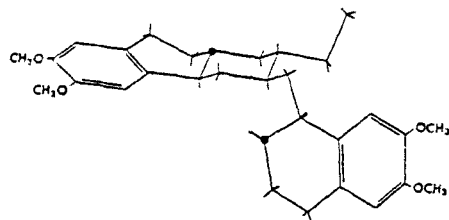
In 1570, a Portuguese monk living in Brazil learned from the natives about the use of the root of *Ipecacuanha* ("little wayside plant that causes vomiting") as a remedy in diarrhea and bleeding.¹ Reports of its use in Europe date as early as the seventeenth century. The son of Louis XIV was successfully treated by Helvetius with his "Brazilian root".² For more than two centuries, ipecac was one of the very few specific chemotherapeutic agents available to pharmacists. A description of the root of *Ipecacuanha* appears in "The Pharmacopoeia of the Royal College of Physicians at Edinburgh", printed in London in 1748 "for John Nourse, at the Lamb, opposite Katherine-Street in the Strand".³ In the 1830 edition of "The Pharmacopoeia of the United States of America", the emetic properties of the root are described as depending "on the presence of a peculiar principle denominated emetine".⁴ The first monograph dedicated to Emetine Hydrochloride, appears in the 1916 revision.⁵

Emetine is the major active constituent of the rhizome and root of *Cephaelis ipecacuanha* and *Cephaelis acuminata* Karsten. It was first isolated in a crude form by Pelletier in 1817 and recognized as an alkaloid in 1823, but the purified alkaloid was prepared only in 1875 (1894 according to another author),⁶ and it was not obtained in crystalline form until 1953.⁷ The first correct empirical formula was reported in 1914,⁸ but the stereochemistry of the four assymetry centers was elucidated only in 1959, after decades of brilliant degradation and synthetic research.⁹

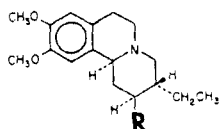
In 1964, Pakrashi isolated emetine and other related alkaloids from the seed kernels and root bark of the Indian plant *Alangium lamarckii* Thwaites, the first reported occurrence of emetine outside the family of Rubiaceae.^{10,11}

Extensive use of emetine in the treatment of amebiasis started in the first quarter of the century. The antiamebic activity and the inhibition of protein synthesis are highly stereospecific and restricted to the natural levorotatory isomer (Fig. 1).

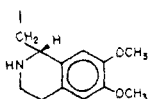
Emetine salts and ipecac (the dried rhizome and roots) are listed in all major national and international Pharmacopeias.¹² Several reviews covering the chemical and biological properties of emetine have been written.^{6,13-17}



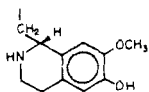
Stereo-formula of emetine. The shaded circles represent N atoms.^{a)}



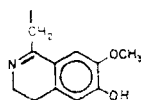
Emetine R =



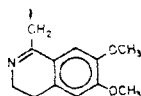
Cephaeline R =



Psychotrine^{b)} R =



O-methylpsychotrine^{b)} R =



Emetamine R =

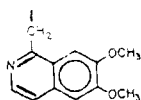


Fig. 1. Emetine and related alkaloids from ipecac.

^{a)} From ref. 13. Reproduced with permission of the copyright owner (Van Nostrand Reinhold Co.).

^{b)} See Section 8 for a discussion on O-methylpsychotrine structure.

2. Description

2.1 Name, Formula, Structure, Molecular Weight

Chemical Abstracts Name Emetan, 6',7'10,11-tetramethoxy, dihydrochloride.

Other Emetine Dihydrochloride, Emetinum Hydrochloridum, Emetini Hydrochloridum, Emetini Chloridum, Ipecine Hydrochloride, Methylcephaeline Hydrochloride, Cloridrato de Emetina.

United States Pharmacopeia definition¹⁸ the hydrochloride of an alkaloid obtained from Ipecac, or prepared by methylation of cephaeline, or prepared synthetically.

Chemical Abstracts Registry Numbers

Emetine dihydrochloride	316-42-7
Emetine	483-18-1

Empirical formula $C_{29}H_{40}N_2O_4 \cdot 2 \text{ HCl}$

Molecular weight 553.57

Structural formula See Fig. 1

2.2 Appearance, Color, Odor, Taste

White, odorless, crystalline powder with a bitter taste. Colorless needles of the heptahydrate (see 5.8) from hot aqueous solution, prisms from concentrated cold solutions.

3. Production

3.1 Extraction from ipecac

Warning against falsifications, the following data were reported in 1945 for the "true" ipecac: total alkaloids 2.00-2.70%, emetine 1.35%, cephaeline 0.25%, psychotrine 0.04%, O-methylpsychotrine 0.015-0.033%, emetamine 0.002-0.006%.¹⁹ Other sources indicate emetine as 50-70% of the total alkaloid content² and the ratio emetine:cephaeline as 2-3:1 in *Cephaelis ipecacuanha* and 1:1 in *Cephaelis acuminata*.²⁰ The USP XX-NF XV definition of ipecac gives the content of cephaeline as varying "from an amount equal to, to an amount not more than twice, the

content of emetine."²¹

Extraction procedures^{6,22,23}

-The alkaloids are extracted with 70% ethanol or methanol (alone or 50%), the concentrated extract is dissolved in water, the solution is made strongly basic with ammonia and extracted with diisopropyl ether. The organic extract is treated with 10-15% aq. KOH to remove cephaeline and evaporated to give emetine, which is purified via the dihydrobromide or dihydroiodide; the halides are converted to the hydrochloride by neutralizing the regenerated free base.

-The Ipecac powder is treated with ammonia, and ether; the alkaloids are extracted from the ether, with diluted H_2SO_4 , the latter is nearly neutralized and washed with ether, then made strongly alkaline and treated with ether (cephaeline remains in the aqueous phase); the residue from the ether solution is taken in methanol and treated with a methanolic solution of HBr to yield emetine hydrobromide.

3.2 Methylation of cephaeline

Cephaeline, which is extracted concomitantly from ipecac, can be converted to emetine with several methylating agents such as: diazomethane, phenyltrimethylammonium hydroxide, dimethyl sulfate, sodium methyl sulfate or nitrosomethylurethane.^{6,22,23} Methylation of the N-atoms by some agents, such as dimethyl sulfate, lowers the yield of emetine.

3.3 Total synthesis

Total synthesis of emetine was undertaken initially to obtain confirmation of the structure and stereochemistry. The first synthesis was completed in 1950²⁴ and although more than a dozen routes were reported¹³⁻¹⁵ using various starting materials and combinations of stereospecific reactions and resolution of racemic mixtures, the research continues, stimulated by the isolation of new structurally related alkaloids and by the search for biologically active synthetic analogs.²⁵⁻²⁹

Fig. 2 summarizes a procedure developed by Openshaw in 1963³⁰ and reportedly used for commercial production by Burroughs-Wellcome.

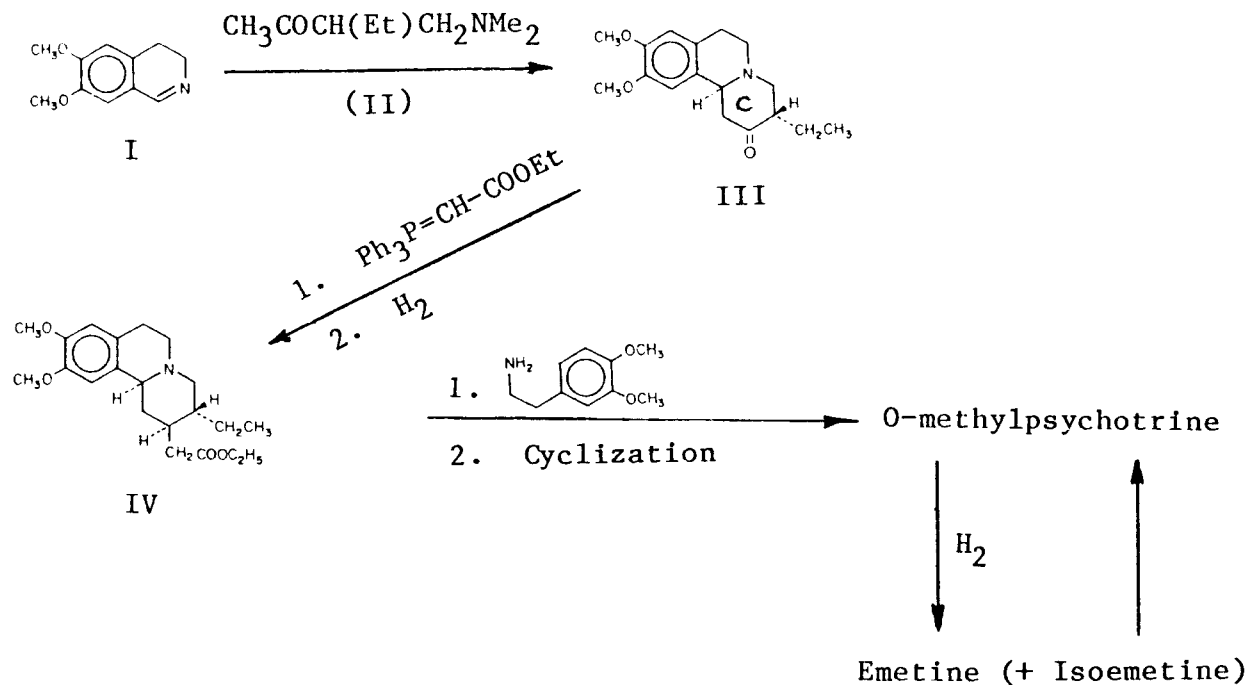


Fig. 2. Openshaw procedure for the total synthesis of emetine.³⁰

The condensation of 6,7-dimethoxy-3,4-dihydroisoquinoline (I) with the Mannich base (II) gives the aminoketone III in almost quantitative yield. A very elegant resolution of racemic intermediate III is obtained by refluxing it in the presence of (-) camphor-10-sulfonic acid, when the desired levo-enantiomer precipitates as a salt and the dextro-enantiomer is racemized by reversible opening of ring C (with continuous precipitation of levo-III, the result is an almost total conversion).

The condensation of III with the Wittig reagent, ethoxycarbonylmethylenetriphenylphosphorane is stereoconservative. Condensation of IV with homoveratrylamine succeeded by Bischler-Napieralski cyclization affords O-methylpsychotrine which is hydrogenated to a mixture of emetine and isoemetine. The yield in emetine is increased by reversion of inactive isoemetine into O-methylpsychotrine by N-chlorination and treatment with base.

4. Physical Properties

4.1 Spectra

4.11 Ultraviolet

The values for the absorption maxima and the absorbances of a 1% solution in a 1.0 cm cell reported in the literature are presented in Table I.

A spectrum of USP Emetine Hydrochloride Reference Standard in water is shown in Fig. 3.

The effect of substituents on the UV spectra of isoquinolinic drugs (including emetine) has been studied.⁴²

4.12 Fluorescence

The spectrum of natural visible fluorescence of emetine in aqueous solutions was found to be analogous to that of other compounds containing one or two o-dimethoxyphenyl groups. Both at pH 1.23 and 4.65-4.70 excitation wavelength maximum is at 360 nm and emission maximum is at 460 nm.⁴³

Other authors reported a fluorescence maximum at 318 nm with an excitation maximum at 284 nm (see 5.6 and 6).

Table I

UV Data for Emetine and Emetine Hydrochloride ^{a)}Emetine

Solvent	λ_{max} , nm	$E_{1\text{cm}}^{1\%}$	Ref.
Chloroform	285	163	31
	290(infl)	148	
	290	210	32
0.1 N H ₂ SO ₄	285	208	32
	281.5	227	33
	285(infl)	208	
4N methanolic ammonia	285	161.5	34
Ethanol	235(sh)	(329)	35
	285	(131)	
	360(sh)	weak	
	283.5	(120)	6
	349.5	weak	
<u>Absolute ethanol</u> ^{b)}	226	(387)	6
	283	(177)	

Emetine Hydrochloride

Water ^{b)}	229	265	36
	283	122	
	279	85	37
Ethanol ^{b)}	230	(250)(238)	38-40
	283	(109)(103)	
0.1 M H ₂ SO ₄	228	-	41
	281	-	

a) The values in parenthesis are calculated from reported log ϵ values.

b) Minimum at 256 nm.

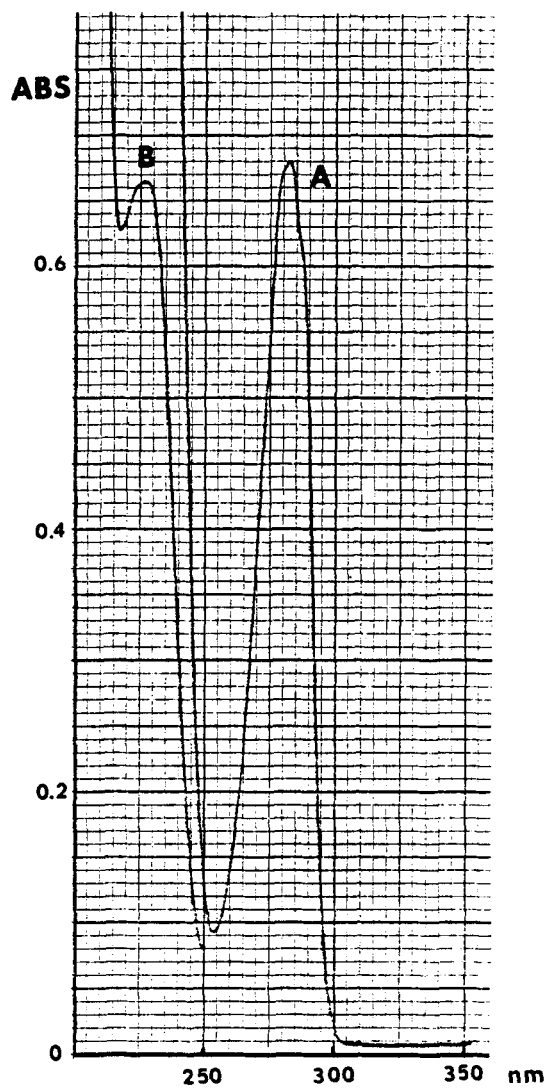


Fig. 3. Ultraviolet Spectrum of Emetine Hydrochloride in Water. A-5.5 mg/100 ml. B-2.2 mg/100 ml. Instrument -- Beckman UV 5260.

4.13 Infrared spectra

The principal peaks in the spectrum of emetine (KBr disk) are at 1514, 1256, 1228 and 1463 cm^{-1} .³³

The presence in the IR spectrum of 2'-benzoyl-emetine of Bohlmann bands around 2740 cm^{-1} confirmed the chemical evidence for a trans-configuration of the quinolizidine moiety (trans-diaxial relationship between the pair of electrons on N-5 and the H atom at 14).⁴⁴

A spectrum of USP Emetine Hydrochloride RS in a KBr disk is presented in Fig. 4. It shows a strong band at $2.93\text{ }\mu$ (H_2O) and a wide band at $3.83\text{ }\mu$ (N^+H).⁴⁰

4.14 Nuclear Magnetic Resonance

An 80 MHz proton magnetic resonance spectrum of emetine in CDCl_3 containing TMS as an internal reference is presented in Fig. 5. It is identical with a previously published spectrum (no assignments).⁴⁵ The signals from aromatic protons in the 6.4-7.2 ppm range and those from the etheric methyl groups around 3.8 ppm are in the expected regions, but the complexity of multiplets from the rest of the non-aromatic protons precludes any practical analytical use.

The 20 MHz proton-noise decoupled ^{13}C spectrum of the same sample is shown in Fig. 6 and the assignments of the chemical shifts, as reported in the literature based upon comparison with related alkaloids,⁴⁶ are tabulated in Table II.

Both spectra were obtained on a Varian FT-80A instrument.

4.15 Mass spectra

The mass spectrum and fragmentation pattern for emetine^{11,47,48} are presented in Fig. 7 and 8.

The mass spectrum in Fig. 7 was obtained by direct-probe introduction of the sample into the ion source of a DuPont 21-492B mass spectrometer operated under data system control (VG 2040, VG Data Systems, Altrincham, Cheshire, England). Operating conditions: resolution, 1000 (10% valley); ion source, 265° ; electron energy, 75eV; ionizing current, 250 μamp ; scan speed, 2 sec/decade.

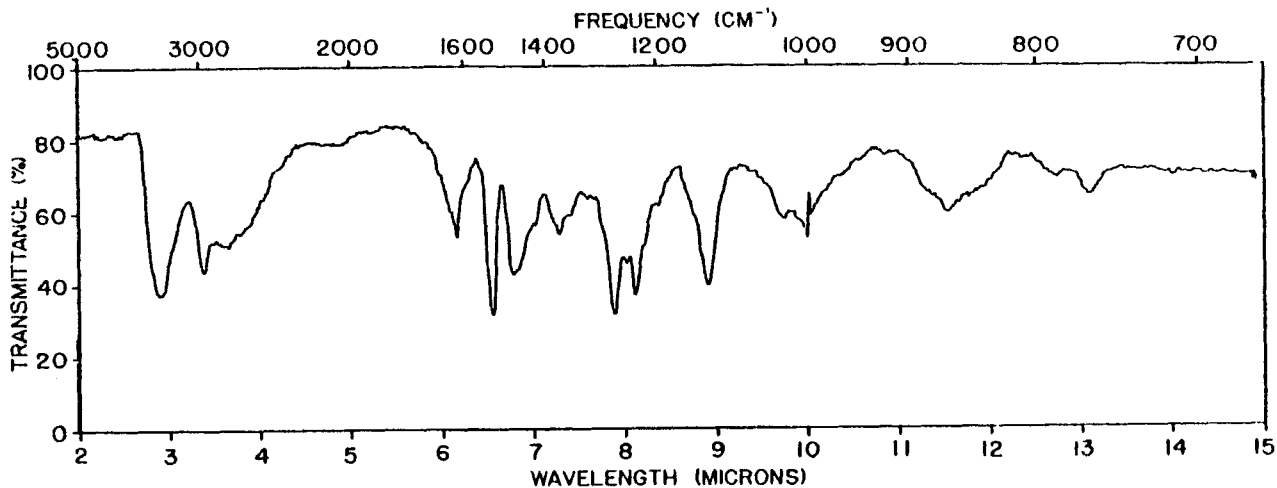


Fig. 4. Infrared Spectrum of Emetine Hydrochloride. KBr pellet. Instrument -- Perkin-Elmer 727.

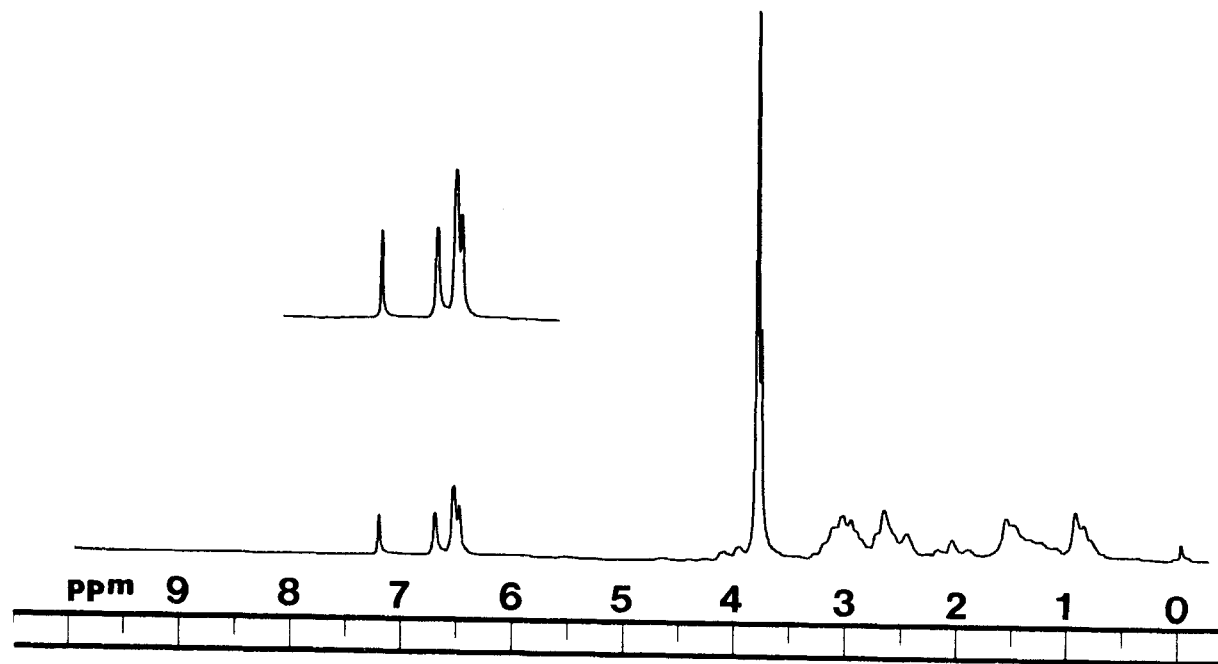


Fig. 5. 80 MHz PMR Spectrum of Emetine in CDCl_3 .

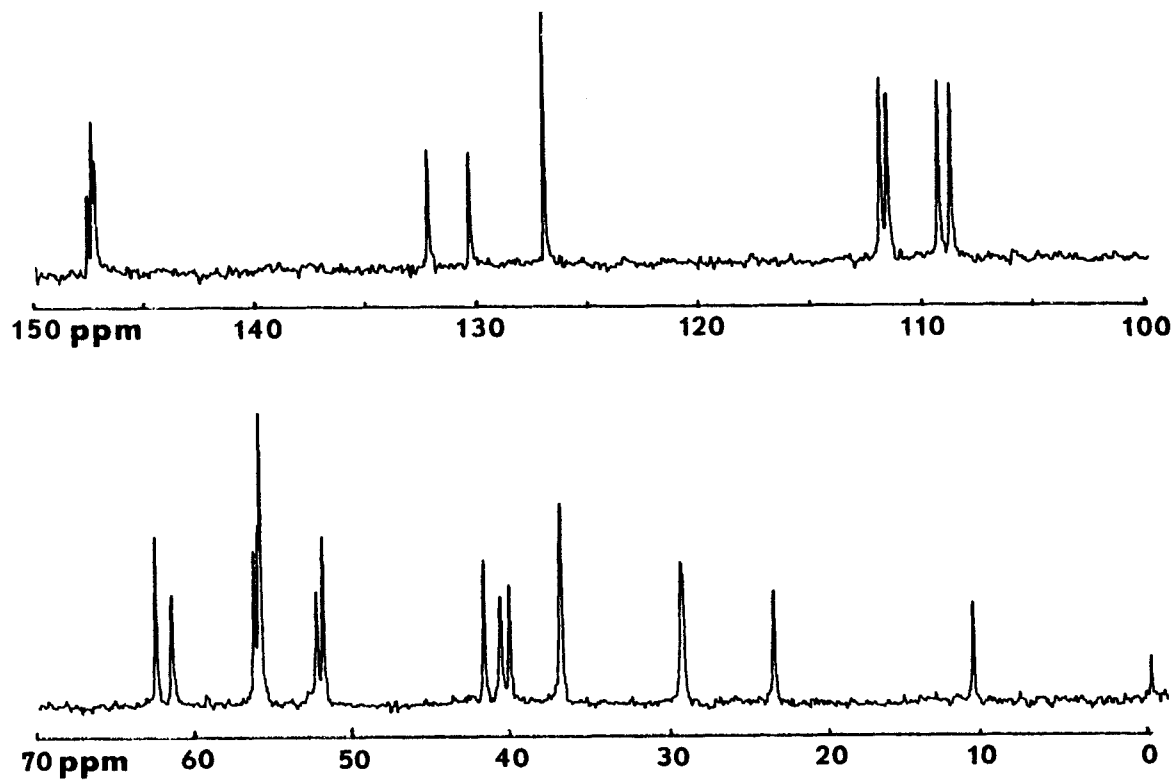
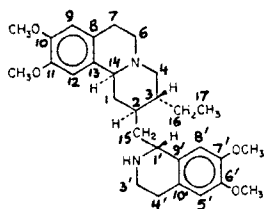


Fig. 6. 20 MHz ^{13}C NMR Spectrum of Emetine in CDCl_3 .

Table II

 ^{13}C Chemical Shifts Assignments for Emetine

Peak no.	C atom no.	Chemical Shift ^{a)}	
		lit ⁴⁶	Found
1	17	10.9	11.2
2	16	23.3	23.6
3	7	29.1	29.4
4	4'	29.1	29.5
5	1	36.7	37.0
6	2	36.7	37.0
7	15	40.0	40.3
8	3'	40.6	40.8
9	3	41.6	41.8
10	6	51.7	52.0
11	1'	52.1	52.4
12	O-CH ₃	55.7	55.9
13	O-CH ₃	55.7	56.1
14	O-CH ₃	55.7	56.1
15	O-CH ₃	55.7	56.4
16	4	61.2	61.5
17	14	62.2	62.5
18	12	108.6	108.9
19	8'	109.1	109.5
20	9	111.5	111.8
21	5'	111.5	112.1
22	8	126.7	127.1
23	10'	126.7	127.1
24	13	130.1	130.4
25	9'	132.0	132.3
26	10	147.1	147.3
27	11	147.1	147.4
28	6'	147.1	147.4
29	7'	147.1	147.7

^{a)} In CDCl_3 solution; in ppm, downfield from tetramethylsilane.

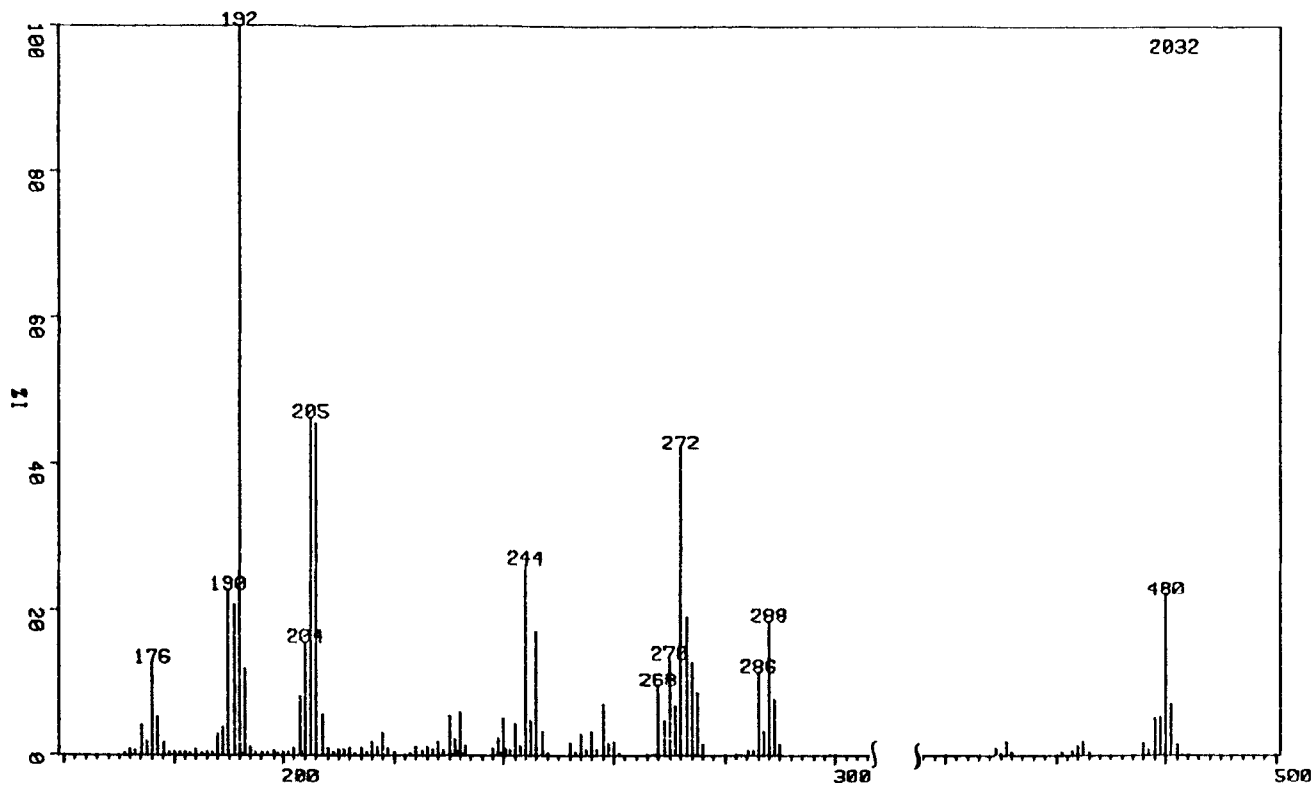


Fig. 7. Mass spectrum of Emetine.

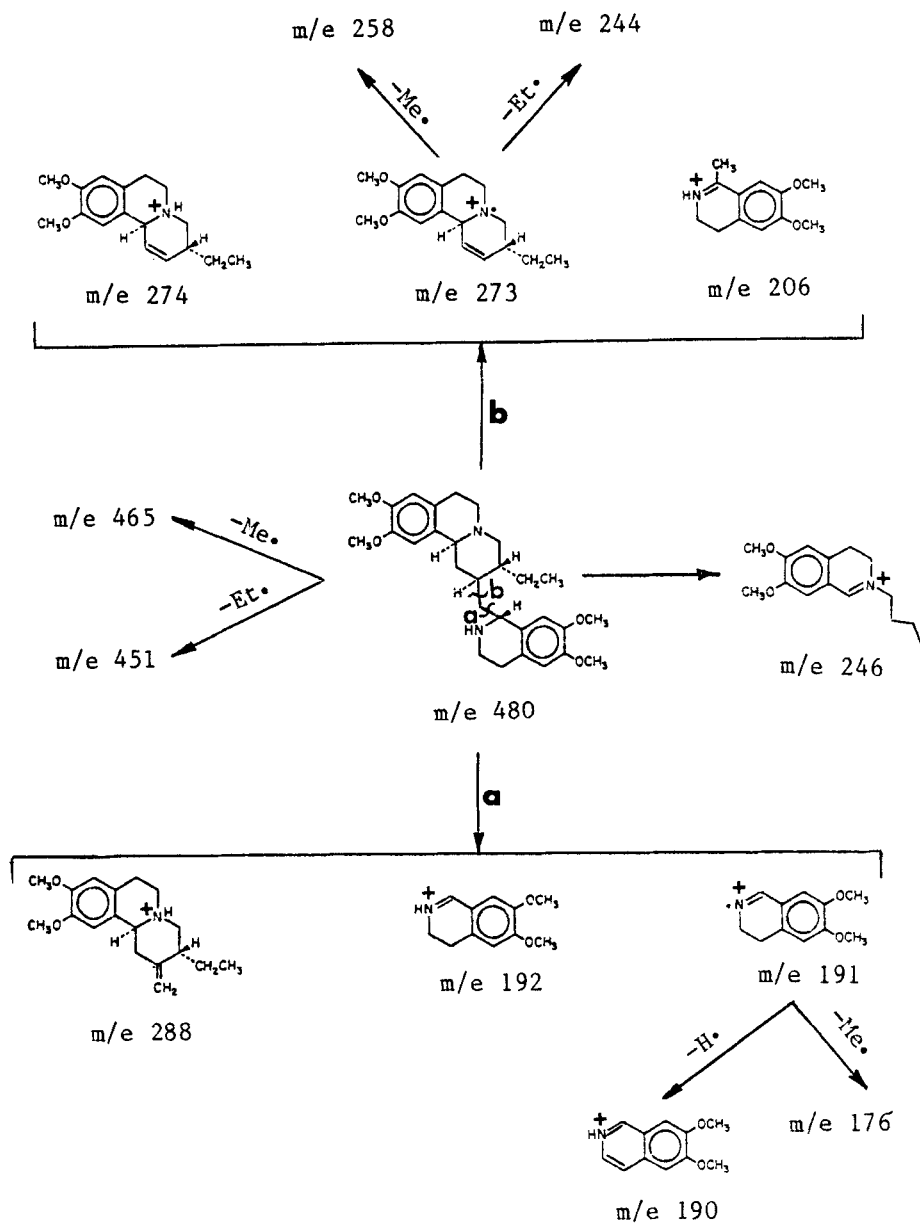


Fig. 8 Fragmentation pattern for emetine

4.2 Melting range

Emetine Hydrochloride contains water of crystallization ranging from 3 to 8 H_2O , depending mainly on the solvent used for crystallization.^{22,49} After drying at 105° , most references indicate a melting range between 235° and 255° with decomposition.⁴⁹ One reference quotes a melting point of $269-270^\circ$ with decomposition.³⁵

The melting point of the amorphous free base is 74° ; crystalline emetine melts at $104-105^\circ$.⁷

In a study of isotonic solutions the freezing-point lowering of a 1% solution of emetine in water was found to be 0.082° .⁵⁰

4.3 Solubility-Partition

Emetine: Water - $0.002\text{g}/100\text{ ml}$ ⁵¹
Conc. NaOH, KOH - insoluble²²
Methanol, ethanol, ether, acetone, acetic acid - very soluble^{22,51}
Chloroform - soluble⁵¹
Benzene - sparingly soluble²²

Emetine Hydrochloride:

Water - $13.1\text{ g}/100\text{ ml}$ (18°)^{22,52}
Diluted HCl - sparingly soluble²²
Ethanol - 1 in 12³³
Chloroform - 1 in 4³³
Ether - insoluble³³

The effect of pH and organic acids on the partition coefficient of emetine was studied.⁵³⁻⁵⁸ The partition coefficient of emetine hydrochloride between chloroform and a mixture water: pH 7.0 0.05 M phosphate buffer 1:1 was 1252.0.⁵⁹

4.4 Dissociation Constants

The following pK_b values have been reported for water:

-at 15° 5.77 and 6.64^{22,60}
-at 40° 5.47 and 6.34⁶¹

Other values reported: 5.73 and 6.74 (no

indication on the temperature)⁵⁹

4.5 Optical Rotation

<u>Emetine</u>	<u>Solvent</u>	<u>g/100 ml</u>	<u>[α]_D</u>	<u>Ref.</u>
<u>Emetine</u>	50% EtOH	1.8	-24.4°	7
			-25.8°	6,16,22
		4.1	-32.7°	6,22
	Chloroform	2	-50°	35
		2.8	-49.2°	62
		3	-49.7°	22
<u>Emetine Hydrochloride</u>	Water	0.9	+11.2°	6,22
		5.0	+17.7°	7
			+17.8°	9
		8.1	+20.9°	6,22
	Chloroform	1	+50.5°	40
			+53°	16,22
	5% HCl	2	+25.7°	22
	Butanol	-	+51°	6
	Benzyl alcohol	-	+17°	6
	Bromoform	-	+83°	6

The study of the optical rotatory dispersion curves of emetine and its salts played a major role in determining the stereochemistry of the molecule.⁹ Since emetine hydrobromide showed no rotational change in the 300-700 nm range, the benzylic centers of asymmetry at C-14 and C-1', the only ones in proximity to UV chromophores, had to be antipodal to one another thus canceling each other's contribution.

5. Methods of analysis

5.1 Identity and Color Tests

Treated with ammonium molybdate or molybdenum oxide in sulfuric acid, emetine gives a bright green color²² (sensitivity 0.1 μ g).³³ The reaction is used in some official compendia as an identification test.^{63,64} Vitali's test yields the following colors: addition of fuming nitric acid - pale yellow; evaporation-pale brown; addition of ethanolic KOH - yellow (sensitivity 1 μ g).³³

An orange-color is produced when emetine is treated in an acidic medium with either H₂O₂^{63,65} or barium

peroxide.⁶⁶ When heated in solid phase with chlgramine, emetine hydrochloride gives a red-yellow color.⁶⁷

Salts of emetine with arylsulfonic acids having characteristic melting points were prepared for identification purposes.⁶⁸

Some of the paper and thin-layer chromatographic separations reported under 5.3 were also recommended as identity tests, preferably in conjunction with colors formed with specific visualization reagents.

The use of the infrared spectrum as an identification test was suggested.⁶⁹

The Identification Tests in the USP-NF monograph of Emetine Hydrochloride call for comparison of IR and UV spectra of the sample with those of USP Emetine Hydrochloride Reference Standard.

All the official compendia require also an identification test for chloride.

5.2 Elemental Analysis

The calculated values for the elemental analysis of emetine hydrochloride are: C 62.91%; H 7.65%; Cl 12.81%; N 5.06%; O 11.56%.⁴⁹

5.3 Chromatographic methods

5.31 Paper chromatography

The paper chromatographic systems have been summarized in Table III.

System No. 1 was reported to separate emetine from some of its stereoisomers, but not from cephaeline.

Emetine is visualized by examination under UV light or spraying with iodoplatinate, bromocresol green, modified Dragendorff reagent or I/KI solutions. When the dried chromatogram is sprayed with a 10% chlorine solution in water acidified with acetic acid, emetine is oxidized to rubremetine and a strong orange-yellow UV fluorescence is obtained (detection limit - 2 μ g).⁷⁸

Table III

Paper Chromatography of Emetine

No.	Support	Developing solvent	Ref.
1	Paper	Ethyl methyl ketone satd. with 2 N HCl	62,70,71
2	---"	BuOH: 0.1 N HCl 1:1	72,73
3	---"	BuOH:MeOH:H ₂ O 45:5:50	74
4	---"	BuOH:AcOH:H ₂ O (various ratios)	72,73,75-79
5	---"	BuOH:formic acid:H ₂ O 120:10:70	72,73,80
6	---"	BuOH:AcOH:AcOBu	81
7	---"	i-BuOH:toluene (satd. H ₂ O) 1:1	74
8	---"	BuOH:AcOBu:Phenol:H ₂ O	81
9	---"	BuOH:Toluene:AcOH:H ₂ O 10:10:5:2	82
10	---"	Acetone:AcOEt:10% NH ₄ OH 2:20:80	83
11	Paper impregnated with 5% sodium dihydrogen citrate	BuOH:aq. citric acid 87:13	33
12	Paper impregn. with phosphate or citrate buffers	CHCl ₃ or trichloroethylene or BuOH saturated with buffers	84
13	Paper impregn. with HCONH ₂	Petr.ether or cyclohexane or CHCl ₃ and Et ₂ NH	85
14	----"----	CHCl ₃ or mixtures with aromatic hydrocarbons	76,86
15	----"----	Triple development: 1) cyclohexane: benzene 9:1 2) benzene 3) CHCl ₃	87

Table III (contd.)

16	Paper impregn. with $\text{HCONH}_2/\text{HCOONH}_4$	CHCl_3 : benzene 9:1	88,89
17	Paper impregn. with tributyrin	pH 7.4 Phosphate buffer	33,90
18	Carboxymethyl- cellulose cation exchange paper	aq. NaCl	91

5.32 Thin-layer chromatography

The chromatographic systems investigated for the analysis of emetine on silica gel plates are presented in Table IV.

The spots can be visualized by one of the following methods:

- spraying with iodine-chloroform solution and heating to 60° for 10-15 minutes: emetine-
lemon yellow (under UV_{365nm}-yellow), cephaeline-
light brown (under UV_{365nm}-light blue)²⁰
- examination under UV_{365nm} (blue)⁹²
- spraying with 10% ninhydrin in 95% ethanol (no color at room temperature) and heating at:
80° (grey-purple), 120° (red-violet) or 160°
(brown-violet)¹⁰⁰
- spraying with 1% chloranil in toluene (brown),
heating to 105° for 15 minutes and spraying with
2 N H₂SO₄ (ochre)¹⁰³
- spraying with Dragendorff reagent (used also on
cellulose or ion-exchange plates)¹⁰⁴⁻¹⁰⁶
- spraying with potassium hexaiodoplatinate (IV)
(K₂PtI₆)^{33,107}

System 12 separates potential impurities present in emetine of natural or synthetic origin: cephaeline, O-methylpsychotrine and isoemetine.

System 7 was reported to separate emetine from its thermochemical and photochemical decomposition products. The procedure was developed into an assay by transferring the emetine spot to a column, eluting with 0.2 N HCl and determining the emetine concentration at 284 nm.

For quantitative purposes, pre-washing of the plates with methanol was recommended.⁹⁴

The chromatographic behavior of emetine on cellulose,^{104,108} Al₂O₃,^{92,109} ion-exchangers,^{105,106} and silica gel-glass powder sintered plates⁹⁹ was also investigated.

Tab. IV

Thin-Layer Chromatography of Emetine

No.	Developing solvent	R _F	Ref.
1	Chloroform:methanol (85:15) or (9:1) ^{a)}	0.3-0.5	20,64,92
2	Benzene:toluene:ethyl acetate:diethylamine:methanol (35:35:20:10:2)	0.54	93,94
3	Toluene:ethyl acetate:85% formic acid (50:45:5)	0	95
4	Toluene:2-propanol:conc. ammonia (70:29:1)	0.26	95
5	Toluene:ethyl acetate:2-propanol:2 N AcOH (10:35:35:20)	0.05	95
6	Toluene:dioxane:methanol:conc. ammonia (25:50:20:10)	0.9	95
7	CCl ₄ :butanol:methanol:ammonia (40:30:30:2) ^{b)}	-	96,97
8	Benzene:ethyl acetate:diethylamine (7:2:1)	0.45-0.64	92,98
9	Chloroform:diethylamine (9:1)	0.67	18,99
10	Ethyl acetate:methanol:conc. ammonia (170:20:10)	0.45	100
11	Methanol:conc. ammonia (100:1.5)	0.52	33
12	Chloroform:2-methoxyethanol:methanol:water:diethylamine (100:20:5:2:0.5)	-	63
13	Chloroform:acetone:methanol (5:4:1)	0.31	101
14	Chloroform:acetone:dimethylformamide	0.52	101
15	0.1 N NH ₃ in Methanol	0.65	80
16	Chloroform:acetone:diethylamine (5:4:1)	0.70	102
17	Methyl ethyl ketone:ethanol:ammonia (5:4:1)	0.70	102
18	Cyclohexane:chloroform:diethylamine (5:4:1)	0.40	92
19	Cyclohexane:diethylamine (9:1)	0.06	92

a) Single or double development.

b) Single or followed by two-dimensional development with petroleum ether: Et₂O:EtOH:Et₂NH (4:16:2:1)

A study of the optimization of the dansylation reaction, TLC separation of mono-dansyl-emetine and fluorescence detection was reported by the same authors¹¹⁰ who later reported a similar HPLC procedure (see 5.34).

Emetine and cephaeline were separated by TLC after a preliminary oxidation by $\text{Hg}(\text{OAc})_2$ to products of characteristic color and fluorescence.¹⁰²

5.33 Gas chromatography

Gas chromatographic methods were described mostly for the screening of toxicological extraction residues.^{35,111-113} As stationary phase 1-5% SE-30 on silanized Gas Chrom P (100-140 mesh) or Chromosorb W (60-80 mesh) was used, with nitrogen or helium as carrier gas; 5-6 feet stainless steel columns were operated at 170-230°. It was reported that apparently emetine hydrochloride dissociated in the injection port (325°) since the same retention times were obtained for the salt and the free base.¹¹¹

5.34 High-pressure liquid chromatography

The systems reported in the literature are summarized in Table V.

Pre-column derivatization:

-dansylation with dansyl chloride; normal phase chromatography (mobile phase--diisopropyl ether:isopropyl alcohol:conc. ammonia 48:2:0.03).¹²¹

Post-column derivatization procedures:

-fluorescence labeling with dansyl chloride in an on-line two-phase "solvent segmentation" flow system (reaction time - 16 min at 56°; excitation 365 nm, emission cut-off filter >450 nm; detection limit 30 ng)

-air-segmented flow, ion-pairing with 9.10-dimethoxyanthracene-2-sulfonate and extraction in chlorinated organic solvents. Excitation at 383 nm, emission - 446 nm. Linearity range 40-600 ng. Limit of detection 0.2 ng.¹¹⁷

Used in conjunction with system 3, the detection limit of a capacitance-conductance detector was 500 ng of emetine.¹¹⁶

Table V

HPLC Analysis of Emetine

Column	Mobile Phase	Ref.
Silica	Ethyl ether (95% water saturated) + 0.5% diethylamine	114
Silica	Chloroform:methanol or ether:methanol	115
Silica	Chloroform:methanol:hexane 7:3:10	116
LiChrosorb RP 8	pH 3.0 0.02 <u>M</u> phosphate buffer:methanol 2:3	117
μ -Bondapak C ₁₈	Methanol:water (56:44) or (60:40) + 0.5% AcOH and 2.5m <u>M</u> octane sulfonate	118
	0.05-0.1 <u>M</u> NaHCO ₃ :acetonitrile 100:0 - 70:30	119
LiChrosorb DIOL	pH 3.0 0.1 <u>M</u> phosphate buffer	117
Mercaptopropyl- bonded phase	Methanol:2 <u>M</u> ammonium hydroxide:1 <u>M</u> ammonium nitrate (27:2:1)	120
Aliphatic strong cation exchanger	Methanol:2 <u>M</u> ammonium hydroxide:1 <u>M</u> ammonium nitrate (27:2:1)	120

5.35 Electrophoresis

The electrophoretic mobility of emetine on paper in buffers from pH 2.3 to 11.4¹²²⁻¹²⁵ and on cellulose-coated glass plates in acidic and alkaline electrolytes¹²⁶ was studied for separation and identification in alkaloid mixtures.

5.4 Titration

Potentiometric titration of emetine hydrochloride with 0.01 N NaOH avoids the difficulties of the visual determination of the end-point due to the buffering effect of the organic base.¹²⁷

Some compendial assays^{63,128} consisted of extraction of an alkaline solution with ether, back extraction of emetine with HCl and titration of the excess of acid. It has been reported that products of photochemical decomposition of emetine interfere with the method.⁹⁷

In other official procedures,^{18,129} emetine hydrochloride is assayed in glacial acetic acid by titration with 0.1 N perchloric acid in the presence of mercuric acetate with crystal violet indicator.¹³⁰⁻¹³² The end-point can be also determined potentiometrically or using p-naphtholbenzein as an indicator.¹³³

Emetine was determined in a two phase chloroform-water system by titration with 0.01 M sodium dioctylsulfosuccinate using dimethyl yellow-Oracet blue as the indicator.¹³⁴

Emetine was radiometrically titrated by ¹³¹I-labeled Dragendorff reagent.¹³⁵

5.5 Colorimetric and Spectrophotometric Methods

Most colorimetric methods for the determination of emetine involve its extraction from aqueous solution into an organic solvent by ion-pairing with a dye anion. Emetine forms a 1:2 complex with bromothymol blue which can be best extracted with chloroform from aqueous solutions buffered in the pH range of 4.0-5.8.^{59,136} Similar procedures were developed using methyl orange, bromocresol purple, bromocresol green, phenol red, Direct Pure Yellow, Acid Blue, cresol red and bromophenol blue.^{53,137-139}

Complications related to the stepwise dissociation of diprotic acids such as bromothymol blue are avoided by using the singly charged tetrabromophenolphthalein ethyl ester. The absorbance of the red extract in 1,2-dichloroethane is measured at 570 nm (linearity range $2-10 \times 10^{-6}$ M (1.1-5.5 $\mu\text{g/ml}$). The method is less pH dependent.¹⁴⁰

The reaction of emetine with sodium 1,2-naphthoquinone-4-sulfonate gives a compound extractable in chloroform and measurable at 460 nm.¹⁴¹

Emetine is precipitated quantitatively from aqueous solutions as a reineckate, which may be dissolved in acetone and determined colorimetrically at 525 nm.^{142,143}

The concentration ranges in which Beer's law is valid for the UV spectrophotometric determinations was reported for 20 quinoline and isoquinoline alkaloids.¹⁴⁴ Emetine acting as electron donor forms a charge transfer complex with iodine whose absorbance in chloroform at 292 nm is greatly increased over that of the uncomplexed alkaloid and can be used for a spectrophotometric assay.^{31,32}

The red shift (322 to 355 nm) accompanying the ionization of picrolonic acid in the presence of emetine was developed in an assay sensitive to 2 $\mu\text{g/ml}$.¹⁴⁵ Emetine forms a colored adduct with picric acid in acetic acid, whose extinction can be measured photometrically.¹⁴⁶

The yellow color produced by the oxidation of emetine with ceric ammonium sulfate was measured photometrically after stabilization with sodium acetate.¹⁴⁷ The colored product resulted from the reaction of emetine with benzoquinone was extracted in CHCl_3 and measured at 540 nm.¹⁴⁸

Emetine hydrochloride yields a highly colored condensation complex (λ_{max} 333 nm) when heated with malonic acid in acetic anhydride. The spectrophotometric method developed on the basis of this reaction has a detection limit of 0.03 $\mu\text{g/ml}$.¹⁴⁹

5.6 Spectrofluorometric Methods

The fluorescence of emetine has a maximum emission at 318 nm with an excitation maximum at 284 nm. Concentrations in the 10^{-6} M range can be determined (as

compared with 10^{-2} M for UV determinations) and the decomposition products seem not to interfere.⁹⁷ The intensity of fluorescence increases linearly over the concentration range 0.01-1.00 $\mu\text{g/ml}$; it increases with decreasing pH being maximal in the 1-3 range and it decreases with the increase of temperature (0.5%/degree in the 15-30° range).¹⁵⁰

Emetine Hydrochloride treated with iodine in alcoholic solution gave a gold-colored fluorescence with λ_{max} at 570 and 620 nm (λ_{ex} 436 nm). Fluorescence intensity was linear with concentration in the range of 0.05-1.00 p.p.m.¹⁵¹

5.7 Polarographic Methods

Emetine yields catalytic waves over the pH range 3 to 10. For quantitative determinations the wave at pH 3 has been employed over the concentration range 0.08-0.25 $\times 10^{-3}$ M (the limiting current is in linear proportion to the concentration) and at pH 8 for the 0.01-0.1 $\times 10^{-3}$ M range (calibration curve necessary).¹⁵² Half-wave potential $E_{1/2} = -1.62\text{V}$.¹⁵³

A polarometric titration of emetine after coupling with p-diazobenzene-sulfonic acid was reported.¹⁵⁴

5.8 Thermogravimetric Analysis

Thermogravimetric analysis at 5°/min in a N_2 atmosphere showed that emetine hydrochloride forms no stable hydrates, water loss takes place even at room temperature (the water content will fluctuate with the relative humidity) and a slow loss continues at temperatures above 105°.¹³³

6. Determination in Biological Fluids and Tissues

In the earlier publications, gravimetric (precipitation with silicotungstic acid; detection limit 20 $\mu\text{g/ml}$),¹⁵⁵ colorimetric (methyl orange or bromophenol blue extraction) and UV spectrophotometric methods¹⁵⁶⁻¹⁵⁸ were used.

The first spectrofluorometric method was reported in 1961.¹⁵⁹ After an extraction procedure adapted to each preparation (plasma, urine, tissue homogenates) a fluorescent compound (rubremetine) is produced by a dehydrogenation reaction with mercuric acetate. ($\lambda_{\text{ex}} = 365$

nm; $\lambda_{em} = 470$ nm).

After extraction by benzene or ether of blood or tissue homogenate samples at an alkaline pH, emetine is taken up in an aqueous acidic solution and determined by measuring the fluorescence at 287-318 nm (sensitivity threshold: 0.01-0.02 $\mu\text{g/ml}$).¹⁶⁰

After extraction with dichloromethane from human plasma, emetine can be analyzed directly at levels above 500 ng/ml by ion-paired reversed-phase chromatography (see 5.34); by introducing an oxidation step with mercuric acetate between extraction and chromatography, the limit of spectrofluorometric detection is lowered to levels of 10 ng/ml of plasma.¹¹⁸

Combustion and liquid scintillation counting were used in pharmacokinetics studies of ¹⁴C-labelled emetine in guinea pigs.¹⁶¹

7. Determination in Pharmaceutical Preparations

Aqueous titration

Depending on the final stage of sample preparation, emetine (or total ether-soluble alkaloids in ipecac) is titrated with 0.01 or 0.1 N HCl or the excess acid used in the final extraction is titrated with 0.02-0.1 N NaOH. The method is used for injections,¹⁸ tablets,¹⁶² ipecac powder or extracts.^{64,73,163-165} The buffering effect of the phenolic alkaloids and the yellow color of the extract tending to mask the end-point have been mentioned as disadvantages of the method when applied to ipecac.¹⁶⁶

Non-aqueous titration in glacial acetic acid with 0.1 N perchloric acid using crystal violet as indicator is used for the assay of emetine formulations.^{130,131} A modified procedure distributes the sample over a mixture of magnesium oxide and Celite and elutes the free base with CHCl_3 .¹³³

Nephelometric¹⁶⁷ and phototurbidimetric¹⁶⁸ titrations were also reported.

Colorimetric and spectrophotometric methods

In 1942 an author was writing "a satisfactory colorimetric method for the determination of [emetine] could not be found...The reaction with hydrogen peroxide in the

presence of hydrochloric acid, which produces an orange colour...is only capable of detecting gross errors in dispensing".¹⁶⁹

The acid-dye technique has been widely used in assaying the alkaloids in emetine formulations, ipecac powder and tinctures.^{140,141,166,170-172} As an example of the technique, the complex formed between the alkaloid and methyl orange at pH 5 is extracted with chloroform and treated with 0.1 N NaOH to liberate the dye and extract the phenolic alkaloids. The liberated dye determined at 460 nm in the alkaline extract is a measure of the total alkaloids. The non-phenolic alkaloids are extracted from the chloroform phase with 0.1 N H₂SO₄ and their concentration in the acid extract is determined at 283 nm and calculated as emetine. The procedure is reported as less time-consuming and requiring less sample than the compendial procedures.¹⁶⁶

After separation from phenolic alkaloids, emetine was assayed in liquid extracts of Ipecacuanha and powdered root by determining the UV absorbance (292 nm) of the charge-transfer complex with iodine.³¹ The yellow color arising by the action of iodine in the presence of aqueous sodium acetate was used previously for the determination of emetine in ipecac and its galenical preparations.¹⁷³

Phosphomolybdic acid was used to precipitate emetine from ipecac extract. The precipitates were taken in acetone and assayed colorimetrically.¹⁷⁴

After buffering the injection at pH 9.5, emetine was extracted in ethylene dichloride and interacted with picrolonic acid. The absorbance at 362 nm (anionic band of picrolonic acid) is used for the assay.¹⁴⁵

Pharmaceutical preparations were subjected to dialysis across a cellophane membrane and the amounts diffusing after a fixed time interval were determined colorimetrically using Lautenschlager's method.¹⁷⁵

Powdered ipecac was extracted with MeOH/HCl, the extract evaporated, mixed with basic Al₂O₃ transferred to a column and eluted with CHCl₃. Emetine was determined as the difference between the total alkaloid content (measured at 286 nm) and the cephaeline content (determined colorimetrically with 2,6-dichloroquinone-chloroimide).¹⁷⁶

Ultraviolet spectrophotometric assays of emetine and cephaeline in ipecac became possible after an elaborate four-column chromatographic system (siliceous earth in different buffers) was developed.^{177,178} The procedure was adopted as an assay for Ipecac and its syrup fluidextract and powdered preparations in the United States Pharmacopeia¹⁷⁹ and the Official Methods of Analysis of The Association of Official Analytical Chemists¹⁸⁰.

Other attempts to extract, purify and separate the alkaloids in ipecac into phenolic and non-phenolic fractions by column chromatography were carried on Fluorisil,¹⁸¹ Al₂O₃,¹⁸² ion-exchange resins,^{34,183} oxycellulose¹⁸⁴ and Celite.¹⁸⁵

After oxidation in aqueous solution with acidic KMnO₄, emetine in pharmaceutical preparations was assayed fluorimetrically.¹⁸⁶

Chromatographic procedures

Thin-layer chromatographic separations followed by densitometric¹⁸⁷ or spot area^{93,188} measurements were used for the quantitative determination of emetine in ipecac and its preparations.

Emetine-containing syrups and capsules were subjected to derivatization with dansyl chloride. Separations carried out by TLC or HPLC were followed by fluorometrical determinations.¹²¹

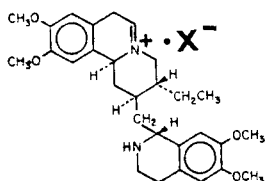
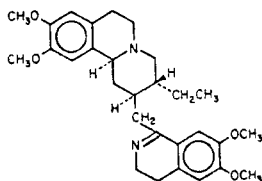
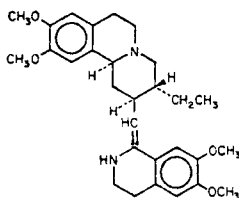
8. Stability - Degradation

Solid forms and solutions of both emetine and the hydrochloride turn yellow on exposure to light and heat. The thermal and photochemical stability of aqueous solutions of emetine hydrochloride are pH dependent,¹⁸⁹⁻¹⁹² pH 3 being that of maximum stability.¹⁸⁹ Cysteine, aminoacetic acid, thioglycolic acid, D-penicillamine, Na₂S₂O₄, NaHSO₃, Na₂SO₃, Pb²⁺ and sodium edetate increased the stability of the solution.¹⁹⁰⁻¹⁹²

The following compounds were identified among the products of the photochemical and thermal decomposition of emetine:emetamine, 3,4-dihydro-6,7-dimethoxyisoquinoline, 0-methyl-psychotrine, 1-methyl-3,4-dihydro-6,7-dimethoxyisoquinoline, tetrahydroemetinium chloride, rubre-metinium chloride, 1',2'-didehydroemetine, 2-methyl-3-ethyl-

1,4-dihydro-9,10-dimethoxybenzo[a]-quinolizinium chloride, 3-ethyl-1,4-dihydro-9,10-dimethoxybenzo[a]quinolizinium chloride and a benzoquinolizinium dimer. The fragmentation products resulted only by photochemical decomposition.¹⁹³

One of the compounds isolated in the above mentioned study, didehydroemetine, was synthesized in 1961 by oxidation of emetine with mercuric acetate and designated by structure A.¹⁹⁴ A recent publication⁴⁸ proved the degradation and synthetic products to be identical, on the basis of their UV, IR and mass spectra and their chromatographic behaviour and assigned to didehydroemetine the structure B. The same paper assigns to O-methyl-psychotrine structure C, instead of the previously reported structure B.

**A****B****C**

9. Toxicity - Pharmacokinetics

In mice, the following acute LD₅₀ values were reported: subcutaneous - 35 mg/kg² (32 mg of base/kg),¹⁹⁵ oral - 30 mg/kg^{33,195} and intraperitoneal - 62 mg/kg.¹⁹⁶ In rats, under intraperitoneal administration, LD₅₀ is 12.1 mg/kg.⁴⁹ The therapeutic dose in men is 1 mg/kg body weight daily, subcutaneously, a course of treatment lasting ten days.¹⁹⁷ The toxic dose by accumulation is between 1.1 and 1.8 g;⁶ 700 mg is considered to be the fatal dose in humans.³³

Emetine is rapidly absorbed and is distributed mainly

in the liver (high concentrations in the mitochondrial fraction), kidney and spleen.^{33,150,156,196,198} Low concentrations were found in the brain, in agreement with unsuccessful attempts to treat amebic cerebral abscess with emetine.¹⁶¹

Emetine does not appear to be metabolically transformed and it is slowly excreted, which may account for the cumulative toxicity (dehydroemetine is eliminated more rapidly). In contradiction with previous findings about emetine being primarily excreted in urine, a 1965 study reported that after intraperitoneal injections of guinea pigs with ¹⁴C-labelled emetine, 95% of the injected radioactivity was recovered from the feces, while only 5% appeared in the urine.¹⁶¹ It was suggested that emetine passes into the bowel through the gastro-intestinal wall where rather high concentrations were found rather than through the bile. In humans, excretion by all other routes than urine is reported to be negligible.¹⁹⁹ Excretion in urine begins 20 minutes after injection and continues for as long as 2 months.³³

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GLIBENCLAMIDE

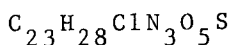
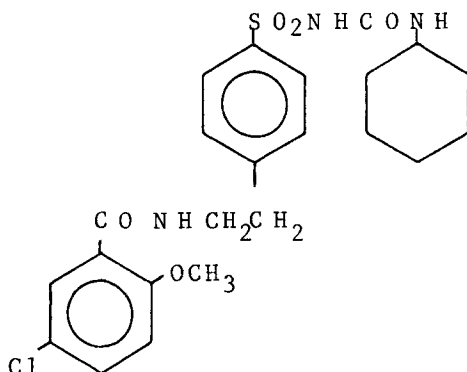
Pamela Girgis Takla

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GLIBENCLAMIDE

1. Description1.1 Name, Formula, Molecular Weight

Glibenclamide is 1-(4-(2-(5-chloro-2-methoxybenz-amido)ethyl)benzenesulphonyl)-3-cyclohexylurea.¹ It is also known² as 5-chloro-N-(2-(4-(((cyclohexylamino)carbonyl)amino)sulphonyl)phenyl)ethyl)-2-methoxybenzamide and as 1-((p-(2-(5-chloro-o-anisamido)ethyl)phenyl)sulphonyl)-3-cyclohexyl-urea.



Molecular Weight = 494.0

Synonyms: Glybenzcyclamide; Glyburide; HB419; U 26,452.

Proprietary Names: Daonil; Euglucon; Diabeta; Maninil;

Lisaghicon; Glidiabet; Euclamin; Gilemal.

1.2 Appearance, Odour, Colour¹

Glibenclamide is a white, crystalline, odourless powder; practically without taste.

1.3 Therapeutic Category

Oral hypoglycaemic.

1.4 Usual Dose Range

2.5 to 20mg once daily.

2. Physical Properties

2.1 Melting Range

This has been reported as 172-174°^{1,3}; 169-170°⁴; and 168-170°⁵.

2.2 Solubility¹

Glibenclamide is virtually insoluble in water and ether; soluble in 330 parts of alcohol, in 36 parts of chloroform, and in 250 parts of methanol. It forms water-soluble salts with alkali hydroxides.

2.3 Infrared Spectrum

Fig. 1 shows the infrared spectrum of a sample of glibenclamide supplied by Hoechst Pharmaceuticals recorded from a potassium bromide disc using a Perkin-Elmer Model 357 grating spectrometer. The spectrum is in agreement with published spectra^{1,6,7}. The major peaks are at 1163, 1333, 1471, 1515, 1613 and 1724 cm⁻¹. According to the findings from a study⁸ of the infrared spectra of a number of sulphonylurea derivatives, assignments for the peaks observed for glibenclamide can be made as follows: 3363 and 3313 cm⁻¹ to urea N-H stretch; 1515 cm⁻¹ to urea, amide II; 1333 cm⁻¹ to -SO₂-N-; 1163 cm⁻¹ (split peak) to -SO₂-. Salt formation has been reported⁹ to decrease the intensity of many of the absorption maxima.

2.4 Nuclear Magnetic Resonance Spectrum

The NMR spectrum (Fig. 2) for glibenclamide in dimethylsulphoxide-D₆ (DMS) was obtained using a Perkin-Elmer R32 (90MHz) spectrometer. The assignments made on the figure agree with those published by Hajdú et al.⁶, who show also the signal produced by the -SO₂-NH proton (offset) at 10.27 ppm. The -CO-NH- proton observed in DMS as a doublet at 6.27 ppm, disappears when the spectrum is determined using trifluoroacetic acid as solvent.

2.5 Ultraviolet Absorption Spectrum^{1,6,7,10}

The ultraviolet absorption spectra for glibenclamide shown in Fig. 3 were determined in 0.01M methanolic hydrochloric acid using 1cm silica cells with a Pye-Unicam SP 1800 spectrophotometer. Absorbance measurements at the wavelengths of maximum absorption were made with a Pye-Unicam

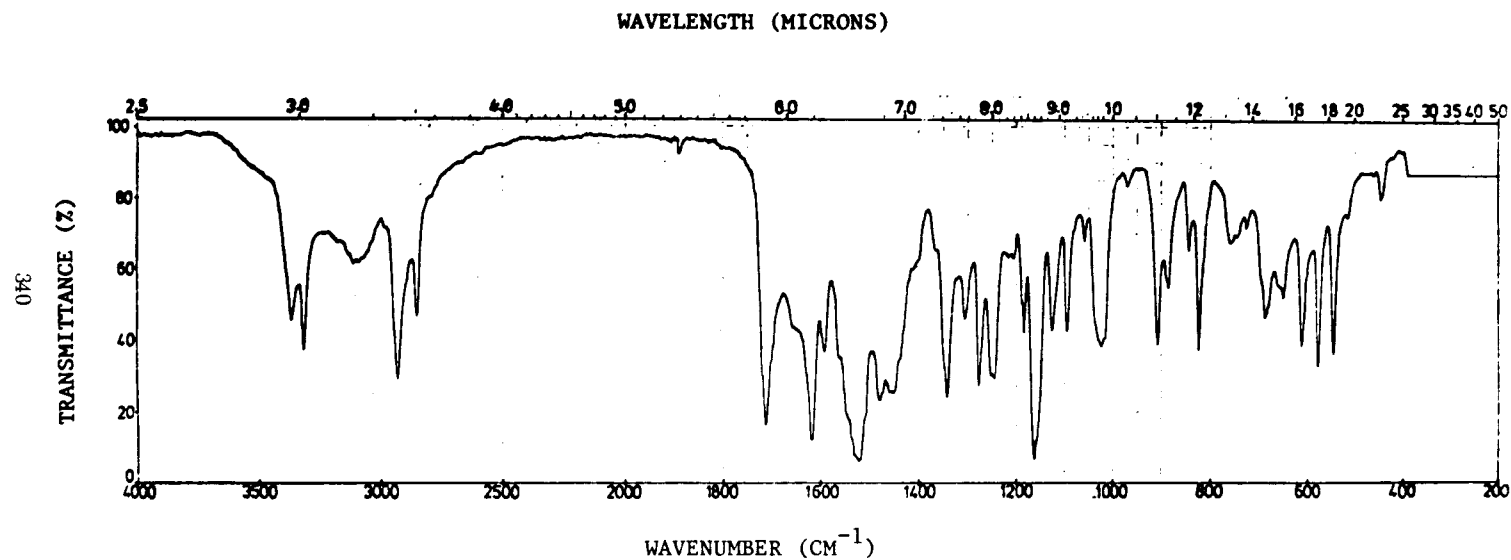


Figure 1. Infrared spectrum of glibenclamide - KBr disc.

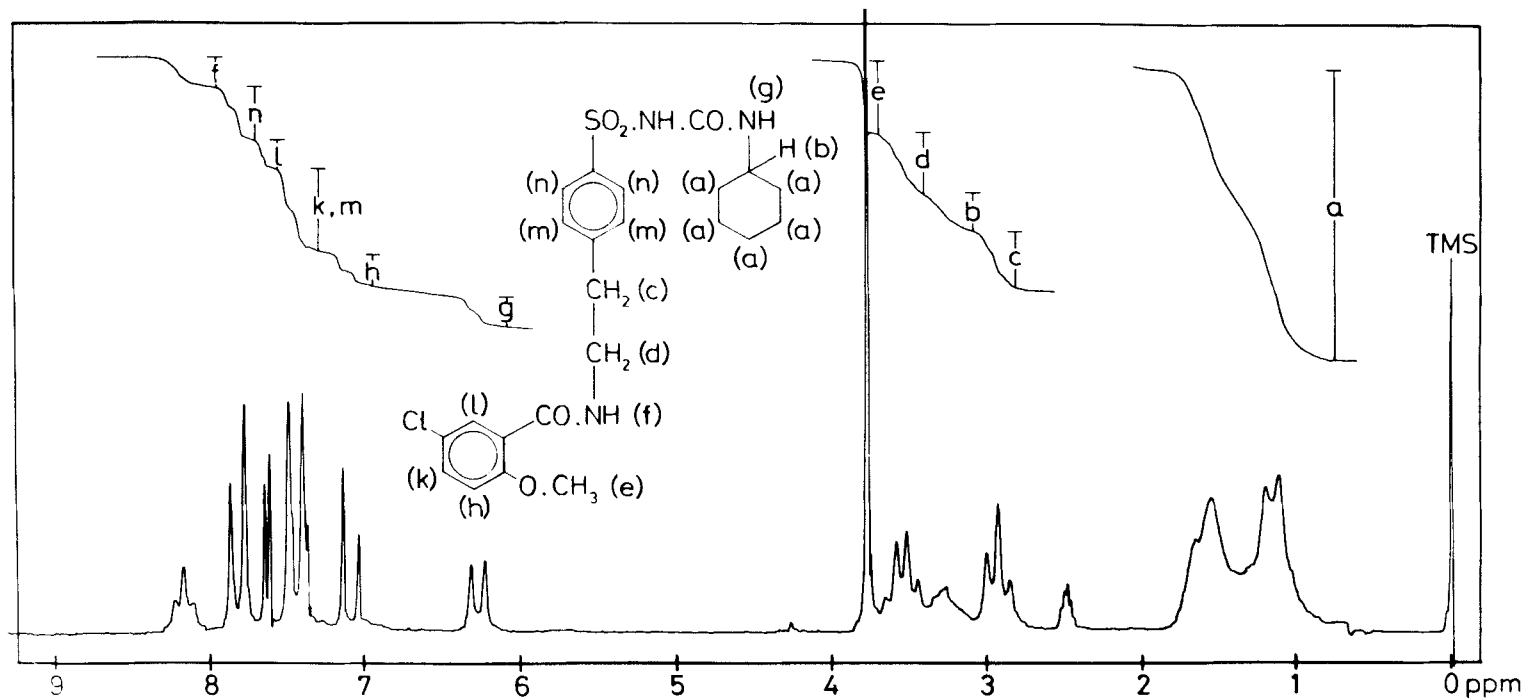


Figure 2. Nuclear magnetic resonance spectrum of glibenclamide in dimethylsulphoxide-D₆.

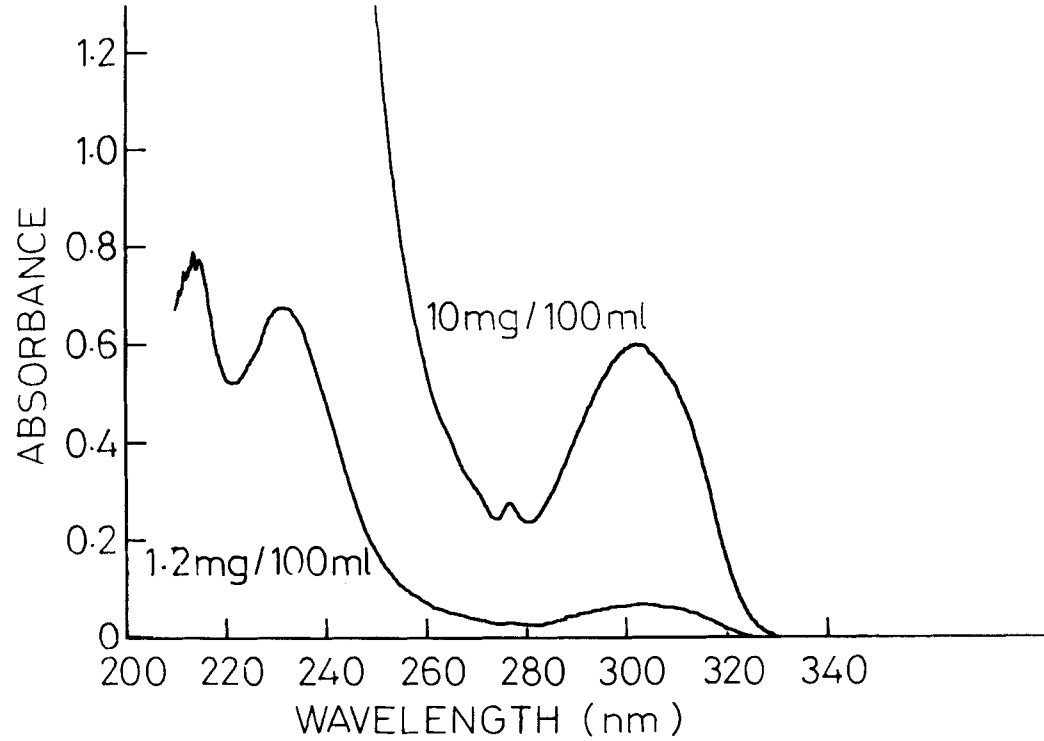


Figure 3. Ultraviolet absorption spectra for glibenclamide in 0.01M methanolic hydrochloric acid.

SP 500 Series 2 spectrophotometer. The sample of glibenclamide used showed negligible loss of weight on drying to constant weight at 105° (less than 0.05% of its weight). The compound shows a characteristic curve with maxima at 229.4nm (A 1%, 1cm about 600), 275nm (A 1%, 1cm 29.6) and 300.4nm (A 1%, 1cm 63.5). In 0.1M sodium hydroxide, the spectrum shows maxima at 226nm (A 1%, 1cm about 480), 274nm (A 1%, 1cm 23) and 300nm (A 1%, 1cm 53).

2.6 Mass Spectrum

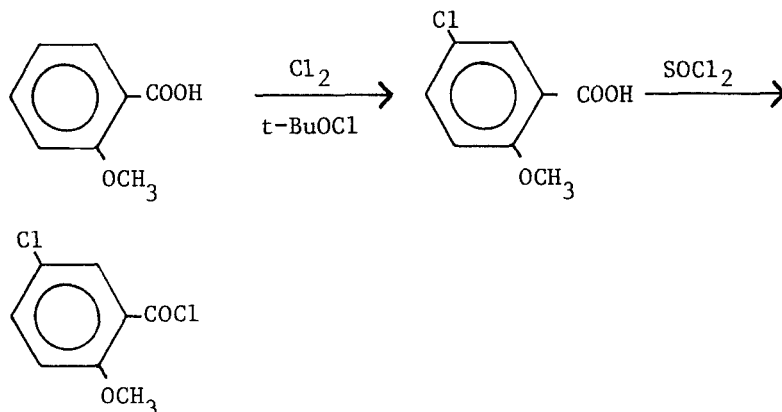
The mass-spectral fragmentation pattern for glibenclamide has been described and discussed by Hajdú et al.⁶, who used a CEC 21-110B instrument to obtain the spectrum. The principal peaks were observed at m/e 394, 368, 352, 288, 198, 169, 125, 99 and 82.

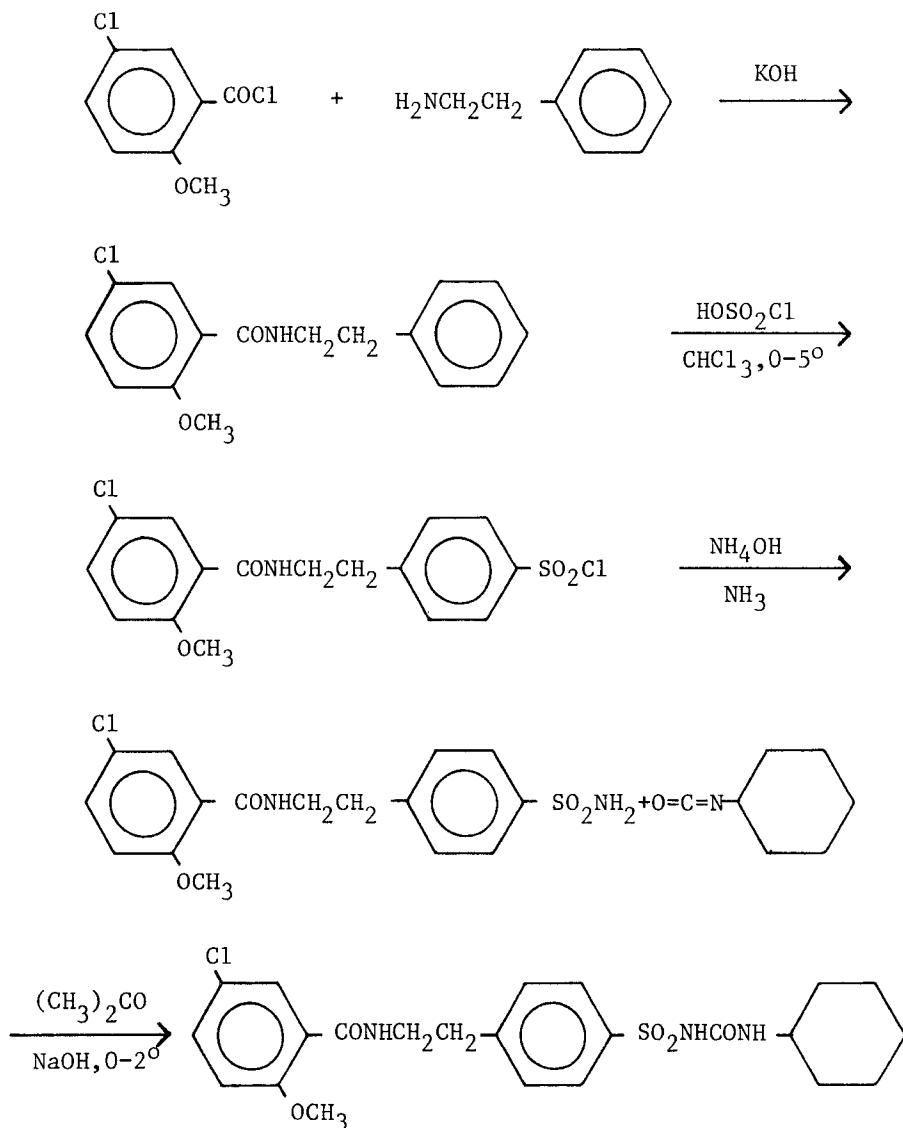
2.7 pKa

Glibenclamide is a weak acid. It has been concluded⁶ that it has the same dissociation constant as tolbutamide (5.3 ± 0.1), since both compounds show the same dissociation at half-neutralisation in solvent mixtures such as methyl cellosolve and water or methanol and water. The direct determination of its pKa in water is impossible owing to its low solubility.

3. Synthesis

Various procedures have been patented for the synthesis^{4,5,11-14} of glibenclamide or its intermediates. The synthesis of glibenclamide has been discussed in a review by Kantolahti and Malkonen¹⁵ who cite the following example¹¹:

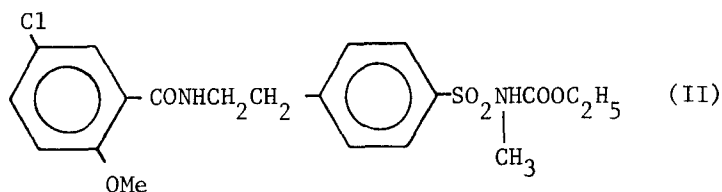
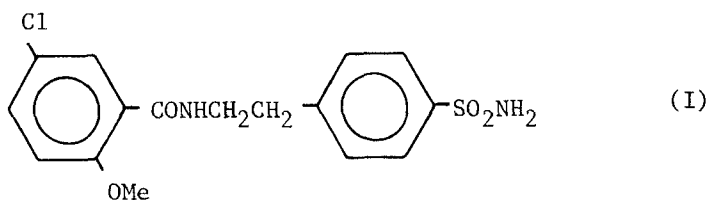




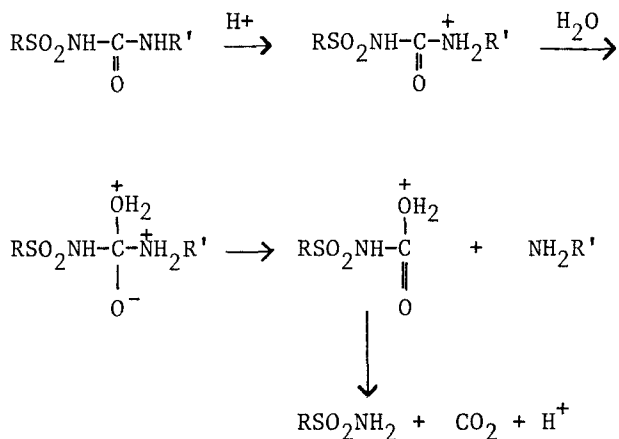
4. Stability

A test is specified in the British Pharmacopoeia 1980¹, using thin-layer chromatography on silica gel GF254 with chloroform-cyclohexane-ethanol-glacial acetic acid (9:9:1:1) as mobile phase, to limit the amounts of 4-(2-(5-chloro-2-

methoxybenzamido)ethyl)benzenesulphonamide (I), ethyl N-4-(2-(5-chloro-2-methoxybenzamido)ethyl)benzenesulphonyl-N-methylcarbamate (II) or related substances which may be present as impurities in glibenclamide or glibenclamide tablets. The spots are observed under an ultraviolet lamp at about 254nm.



Wiseman et al.¹⁶ in a study of sulphonylurea hypoglycaemic agents have postulated that an initial protonation is probably the rate determining step in the hydrolysis of sulphonylureas as follows:



Thus, in the case of glibenclamide, the hydrolysis products would be (I) and cyclohexylamine. Kuriki et al.¹⁷ have described a procedure for the determination of glibenclamide and its decomposition products, in which cyclohexylamine is first extracted into isoamylacetate from aqueous alkaline solution, and determined by a colorimetric procedure based on the Spingler method¹⁸ of assay using 2,4-dinitrofluorobenzene (DNFB). The aqueous solution is subsequently acidified and extracted into organic solvents to allow the determination of glibenclamide by heating with DNFB to produce a yellow colour, and the determination of glibenclamide plus (I) by ultraviolet spectrophotometric measurement at 299nm. Poirier et al.¹⁹ have reported that (II) forms gradually from glibenclamide in methanol or chloroform-methanol (1:1) even at room temperature, and observed that the British Pharmacopoeia test for impurities should be completed immediately after the test solution (in chloroform-methanol) has been prepared. The characterization and structure of (II) formed by refluxing glibenclamide with methanol has been proved by synthesis²⁰. There are no reports that glibenclamide shows instability under normal storage conditions. A report of loss of strength in tablets stored for six weeks at 20° and 75% relative humidity has however been made.²¹

5. Drug Metabolism and Pharmacokinetics

Since plasma levels of glibenclamide are generally low, most metabolic studies have been carried out using the ¹⁴C labelled drug, although in some recent work radioimmunoassay has been used. Pharmacokinetic parameters have been estimated from a single compartment model from investigations in animals^{22,23} and in man^{24,25}. The closest similarities with man were observed in the rabbit. In man, 45% of a single oral dose of 5mg was absorbed, and peak blood concentrations of $0.044 \pm 0.004 \mu\text{g per ml}$ (0.089 ± 0.008 nanomoles per ml) were attained. Other studies^{26,27} however revealed a practically complete intestinal absorption, and it has been shown²⁸⁻³⁰ that the bioavailability of glibenclamide is dependent upon particle size. Fuccella et al.³¹ reported that absorption was complete within 30 to 60 minutes after administration of a 5mg tablet containing micronized glibenclamide. Maximum plasma concentrations^{24-26,31,32} are usually attained within 2 to 4 hours, and are in the range 120 to 360ng per ml after a single 5mg oral dose. Ko et al.³³ found peak levels to occur within 3 to 8 hours. Dose response curves show that the decrease in blood sugar which occurs is limited, and

higher doses only increase the duration of effect³⁴. After a single intravenous injection, the initial biological half-life period was 23 minutes, but the half-life under steady state conditions was 6.6 hours²⁴. The increase in half-life which occurs with time makes it difficult to predict drug levels after multiple doses³². The drug is widely distributed throughout the body, and does not accumulate in the blood. Its apparent volume of distribution, owing to its lipophilic nature, is 10 to 11 litres³¹. It shows no substantial binding to blood cells, but is more than 99 per cent bound to serum proteins²⁴. The binding of glibenclamide to plasma has been studied extensively by equilibrium dialysis^{31,35} and by a fluorescence probe technique³⁶. Brown and Crooks have studied the effect of different salts and buffers³⁷ and of various anionic drugs³⁸ on the binding, which was found to occur by a non-ionic mechanism. No Cotton effects are generated by glibenclamide bound to albumin³⁹. The absorbed drug is completely metabolized, 95% of a single oral dose being excreted within 5 days in similar amounts in urine and faeces^{23,31}. Metabolism probably takes place in the liver³². Metabolites of glibenclamide are formed by hydroxylation of the cyclohexyl ring at positions 3 and 4 to give 4-trans-hydroxyglibenclamide (the principal metabolite) and 3-cis-hydroxyglibenclamide. Both metabolites have been identified in blood, but were without hypoglycaemic effect at the levels found. A third metabolite has been found in trace amounts in urine, but not identified^{24,32}. 4-Trans-hydroxyglibenclamide is about 5 or 6 times less effective than glibenclamide in the rat⁴⁰⁻⁴², and the metabolites are eliminated rapidly with a half-life of 12 minutes provided renal function is normal⁴³. Attempts have been made to fit the disposition of glibenclamide in man³¹ and in the dog⁴⁴ into a two-compartment model with a first-order absorption rate. In another pharmacokinetic study, Balant et al.³² have made a detailed comparison of their findings with other published results, and found that the kinetics involved were too complicated to be resolved adequately by such a model. They suggested instead a third hypothetical slowly equilibrating "deep" compartment, in which the drug could accumulate during long term therapy. Further evidence in favour of this approach is cited in a later report⁴⁵ for which radio-immunoassay was used to measure glibenclamide levels. Comparative studies of the metabolic parameters of various sulphonylureas including glibenclamide have been made in rabbits⁴⁶ and in man⁴⁷. Happ et al.⁴⁸ measured glibenclamide levels by radioimmunoassay in a comparative study in adult diabetics. Some reviews on glibenclamide metabolism have been published⁴⁹⁻⁵¹.

6. Methods of Analysis

6.1 Polarography

Procedures have been described by Silvestri^{52,53} and by Tammilehto⁵⁴. For quantitative work, an automated system, having a flow through micro cell used with a silver-silver chloride reference electrode, has been stated⁵³ to give good reproducibility.

6.2 Non-Aqueous Titration

Tetramethylurea has been used as solvent for the titration of glibenclamide with 0.1 N lithium methoxide in benzene-methanol.⁵⁵ The end-point was determined potentiometrically or by using 0.2% azoviolet in toluene as visual indicator. Tablet excipients generally were found not to interfere with the assay. Alternatively, the assay can be performed by titration with 0.1 N potassium hydroxide in dimethylformamide solution with thymolphthalein as indicator⁵⁶.

6.3 Chromatography

Several procedures^{6,57-62} have been proposed for the identification of glibenclamide by thin-layer chromatography. Among the solvent systems described are butanol-methanol-chloroform-25% ammonia⁵⁸, propanol-cyclohexane⁵⁹, and propanol-benzene-cyclohexane⁵⁹.

High-performance liquid chromatography has been recommended by Beyer⁶³ for the quantitative determination of glibenclamide in tablets. The column packing used was 1% ethylene propylene copolymer on DuPont Zipax, with 0.01 M sodium borate containing 27.5% v/v methanol as mobile phase. Testosterone serves as internal standard. An impurity, 5-chloro-N-(p-sulphamoyl-phenethyl)-o-anasimide, was eluted as a separate peak.

7. Identification and Determination in Pharmaceuticals

Identification tests for glibenclamide given in the British Pharmacopoeia¹ depend upon: a) its infrared absorption spectrum; b) its light absorption in the range 230 to 350nm; c) the evolution of fumes having a pungent, amine-like odour which change moistened red litmus paper to blue after boiling with 6M sodium hydroxide solution; and d) positive tests for chloride and sulphate in an aqueous

extract of the residue obtained after igniting glibenclamide with anhydrous sodium carbonate and potassium carbonate.

The identification tests for glibenclamide in tablets depend upon: a) light absorption measurements in the range 230 to 350nm; and b) thin-layer chromatography on silica gel GF254 with chloroform-cyclohexane-ethanol (96 per cent)-glacial acetic acid (9:9:1:1 parts by volume) as mobile phase.

Glibenclamide is assayed¹ by titration in ethanol with 0.1M sodium hydroxide using phenolphthalein solution as indicator, and protecting against exposure to atmospheric carbon dioxide. Glibenclamide tablets are assayed¹ by a spectrophotometric procedure which depends upon extraction of the tablets with 0.1M methanolic hydrochloric acid, and measurement of absorbance at about 300nm.

8. Identification and Determination in Body Fluids

8.1 Extraction

Glibenclamide is extracted from aqueous acid solution or acidified plasma or serum by chloroform^{6,64,65}, ethyl acetate⁶, amyl acetate⁶⁶, toluene⁶⁷ and benzene⁶⁸. Alternatively³¹, plasma can be deproteinized with acetone, the acetone evaporated to small volume and extracted with chloroform after dilution with pH 4.5 buffer solution. Balant et al.³² were not successful in separating glibenclamide from its metabolites using a procedure that they had found applicable to glipizide which involved adjusting the pH of plasma to 4.3 with acetate buffer, and extracting with methylene chloride.

8.2 Ultraviolet Spectrophotometry

A procedure for glibenclamide in serum has been described by Hajdú et al.⁶, but is insufficiently sensitive for normal applications.

8.3 Colorimetry

A modification⁶ of the colorimetric procedure reported by Spingler¹⁸ for tolbutamide in serum involves heating glibenclamide in amyl acetate with 2,4-dinitrofluorobenzene to 150° for 5 minutes. Absorbance is measured at 380nm. The method can only be used for glibenclamide when it is present in much higher concentrations than those normally encountered in serum.

8.4 Fluorimetry

Glibenclamide when excited by radiation of wavelength 290nm emits a weak fluorescence which can be measured in 0.1 M sodium hydroxide at 350nm. Hajdú et al.⁶ have described a procedure for serum, but the method has never been successfully applied. The detection limit for glibenclamide in aqueous alkali is about 0.4 μ g per ml, and plasma blanks are likely to be high⁶⁹. Becker⁶⁶ has reported that his fluorimetric procedure which was developed for glibornuride in plasma can be applied to glibenclamide. The lower limit of detection for glibenclamide is about 40ng per ml. The fluorescence is developed by heating an amyl acetate extract of the drug at 140° for 15 minutes with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride). The reaction depends upon the degradation of glibenclamide to give 4-(2-(5-chloro-2-methoxybenzamido)ethyl)benzenesulphonamide and cyclohexylamine. The latter compound reacts with NBD chloride present in excess to produce a fluorescent product. The reagent itself is non-fluorescent. There are no other reports of this method having been used for the determination of glibenclamide.

8.5 Thin-layer Chromatography

Balant et al.³² used silica gel plates with benzene-glacial acetic acid-ethyl acetate-acetone (65:6:12:30) for methylene chloride extracts of urine and plasma.

8.6 Gas-Liquid Chromatography

A procedure⁶⁵ employing a column packed with 5% OV-17 on 80-100 mesh Chrom G-AW-DMCS has been used for the determination of glibenclamide in the plasma of healthy adults after oral administration of 5mg of the drug. The method involves derivatization of the glibenclamide by heating with 2,4-dinitrofluorobenzene in amyl acetate at 130°C for 1 hour. A ⁶³Ni electron-capture detector was used. The procedure was found to be specific for glibenclamide, and not subject to interference by metabolites. 4-Hydroxyglibenclamide could be determined qualitatively by a slight modification of the gas chromatograph parameters. The quantitative determination of glibenclamide was carried out using tolbutamide as internal standard. The lowest detectable amount of glibenclamide was 100pg. Plasma concentrations found 1, 3 and 5 hours after the administration of the drug are reported. They range from 0.05 to 134.76 ng per ml.

8.7 High-Performance Liquid Chromatography

Several procedures have been reported in recent months^{67,68,70} which are sufficiently sensitive for clinical assays. Adams and Krueger⁶⁷ mix canine serum with monobasic sodium phosphate solution and extract with toluene containing butyl-p-hydroxybenzoate as internal standard. The extract is evaporated to dryness, and the residue is dissolved in the h.p.l.c. mobile phase which is 50mM-NH₄H₂PO₄-acetonitrile (1:1). Chromatography is carried out on a reversed-phase column of Lichrosorb RP-8. Detection is at 228nm. The lower glibenclamide detection limit was about 20ng per ml of serum extracted. The major metabolites in the dog, 3-cis-(1-((4-(2-(2-methoxy-5-chlorobenzamido)ethyl)phenyl)sulphonyl)ureido)cyclohexanol, 1-((4-carboxyphenyl)sulphonyl)-3-cyclohexylurea, and 2-methoxy-5-chlorobenzamide did not interfere. The main metabolites of glibenclamide in human serum are also stated not to interfere. Another procedure⁶⁸ developed for glipizide which has been found applicable also to glibenclamide uses a μ Bondapak C₁₈ column. Glibenclamide was extracted from serum with benzene after acidification to pH3. The mobile phase was 30% 0.01 M phosphate buffer (pH 3.5) in 70% methanol. Glibornuride served as internal standard. Reinauer et al.⁷⁰ used an RP18 column with CH₃CN-H₃PO₄ (45:55) as mobile phase for the determination of glibenclamide in blood serum of diabetics.

8.8 Radioimmunoassay

A number of radioimmunoassays have been developed which have the desired sensitivity for metabolic studies. Some of these⁷¹⁻⁷³ do show cross-reaction with the two major metabolites of glibenclamide. The radioimmunoassay developed by Kawashima et al.⁷⁴ is however stated not to be subject to interference from these metabolites, although the closely related hypoglycaemic drug, glipizide, does show significant cross reactivity. The antiserum is produced in rabbits immunized with an antigen prepared by conjugating the diazonium salt of N-(p-amino-benzamidoethyl)-benzenesulphonyl-N'-cyclohexylurea to bovine serum albumin through diazo-coupling. Dextran coated charcoal is used to adsorb the free glibenclamide, and separate it from the bound drug. It was found possible, with this procedure, to determine as little as 2.5ng per ml of glibenclamide in plasma by using 10 μ l samples without the need for extraction. Results obtained with dog plasma samples were comparable with those obtained by the less sensitive liquid chromatography method. The paper gives also the results of plasma assay carried out in diabetic

patients on glibenclamide treatment. A patent has been taken out in connection with this procedure⁷⁵. Lindner et al.^{76,77} have compared the determination of glibenclamide in the serum of diabetics by radioimmunoassay and high-pressure liquid chromatography.

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HEROIN

Dorothy K. Wyatt and Lee T. Grady

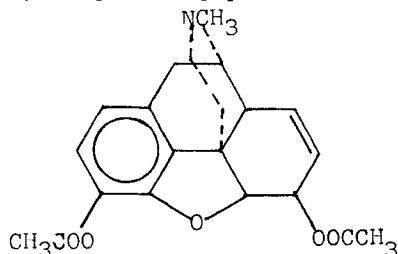
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1. Description

1.1 Heroin

1.1.1 Name, Formula, Molecular Weight

Heroin is 3,6,-diacetoxy-7,8-dehydro-4,5 epoxy-N-methylmorphinan [4]. The CAS registry no. is 561-27-3 [1].



$C_{21}H_{23}NO_5$
molecular weight 369.4

1.1.2 Appearance, Color, Odor

White crystals which turn pink and emit an acetic odor on prolonged exposure to air [2].

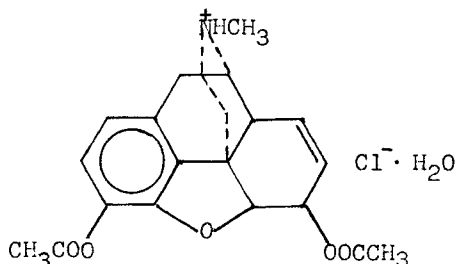
1.1.3 Synonyms [2]

Acetomorphine
Diacetylmorphine
Diamorphine
7,8-Didehydro-4,5 α -epoxy-17-methylmorphinan-3,6 α -diol diacetate (ester)

1.2 Heroin Hydrochloride

1.2.1 Name, Formula, Molecular Weight

Heroin hydrochloride is 3,6-diacetoxy-7,8-dehydro-4,5 epoxy-N-methylmorphinan hydrochloride monohydrate [4]. The CAS registry no. is 1502-95-0 [1].



$Cl^- \cdot H_2O$

$C_{21}H_{23}NO_5 \cdot HCl \cdot H_2O$
molecular weight 423.9

1.2.2 Appearance, Color, Odor

The hydrochloride is an almost white, crystalline powder, odorless when freshly prepared but develops an odor characteristic of acetic acid on storage [50,51].

1.2.3 Synonyms

3,6-di-O-acetylmorphine hydrochloride monohydrate [50]
Diacetylmorphine hydrochloride [50]
Diamorphine hydrochloride [50]
7,8-Didehydro-4,5 α -epoxy-17-methylmorphinan
3,6 α -diol diacetate (ester) hydrochloride monohydrate [2]

2. Physical Properties

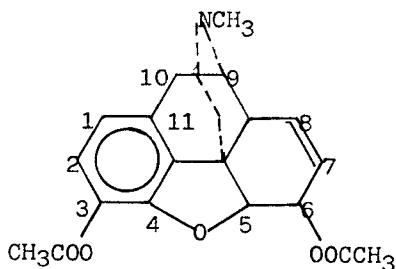
2.1 Infrared Spectra

The infrared spectra are presented in Figure 1. The spectra were obtained from potassium bromide and potassium chloride dispersions of previously dried material (105°, constant weight) using a Beckman 5260 grating infrared spectrophotometer. Principal bands are 1765, 1740, 1450, 1370, 1250, 1180 cm^{-1} [61].

2.2 Nuclear Magnetic Resonance Spectra

2.2.1 Proton Spectrum

The proton spectra are presented in Figure 2.



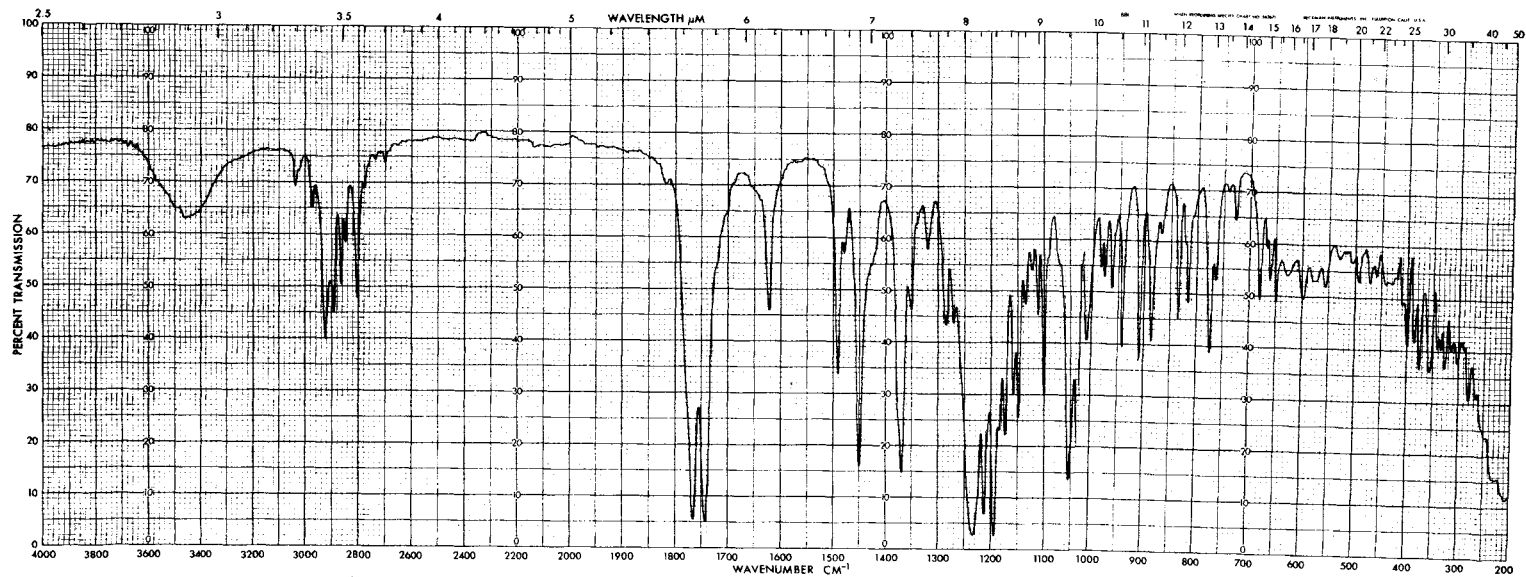


Figure 1a: Infrared spectrum of heroin.

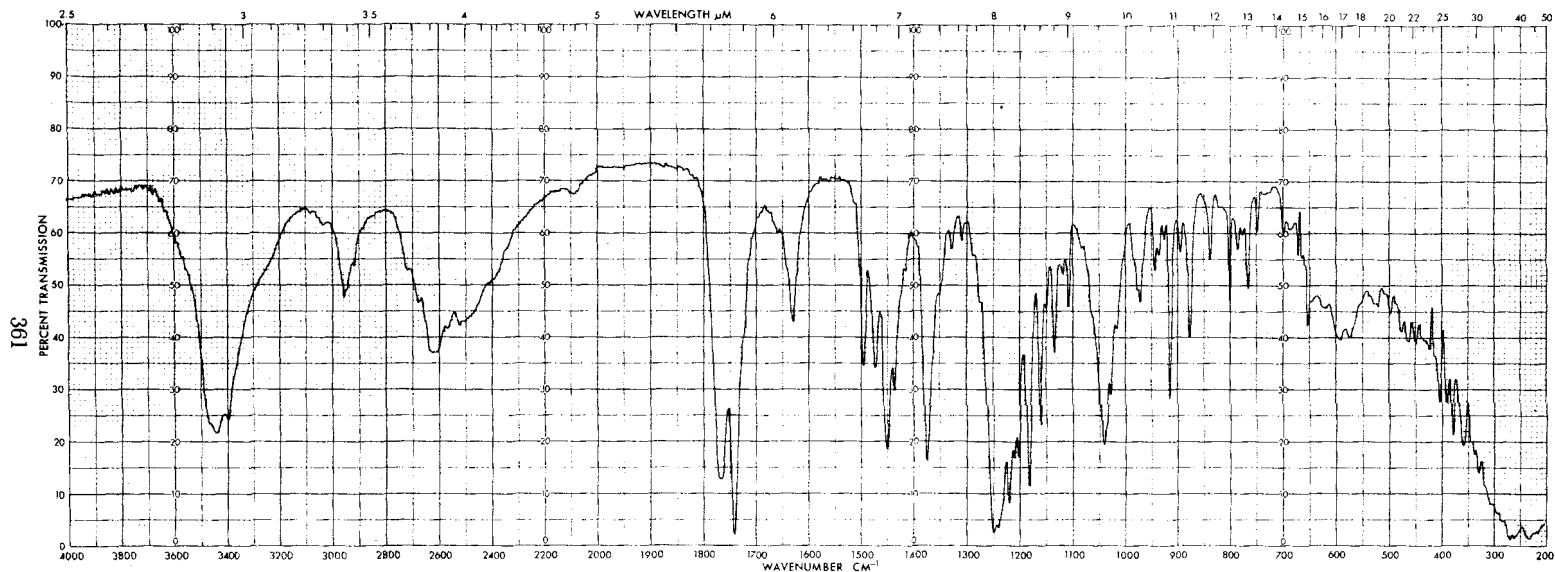


Figure 1b: Infrared spectrum of heroin hydrochloride.

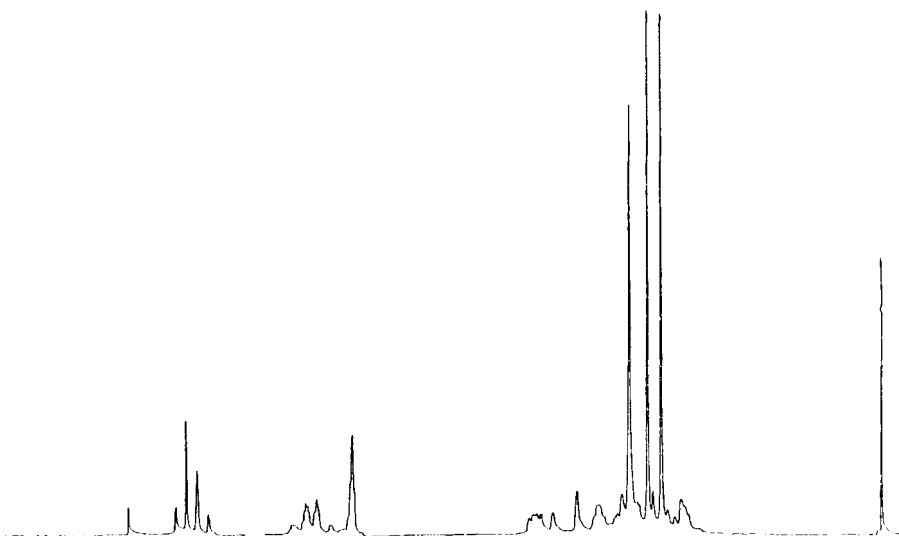


Fig. 2a. Proton NMR spectrum of heroin base.

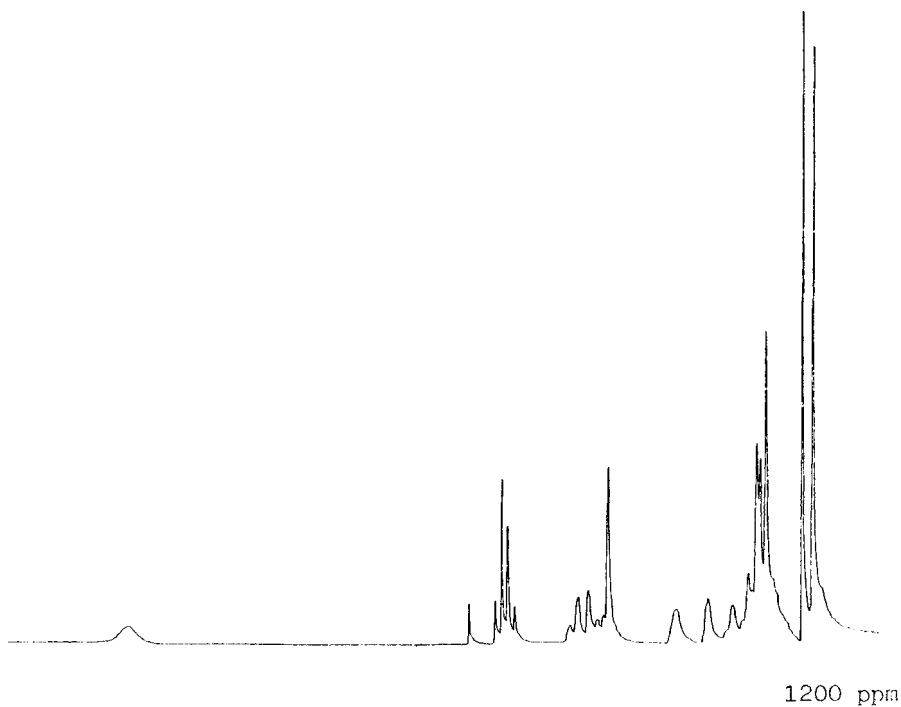


Fig. 2b. Proton NMR spectrum of heroin hydrochloride.

Spectral assignments are listed in Table I

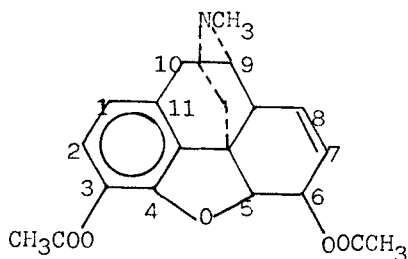
Table I

^1H NMR Spectral Assignments for Heroin [48]

<u>Chemical Shift</u> <u>ppm (δ)</u>	<u>Multiplicity</u>	<u>Characteristic of proton</u>
2.27, 2.11	s	acetyl
2.88	s	N-methyl
3.33	m	10
3.70	m	9
5.45	d	8
5.21	m	5,6
5.73	d	7
6.87	d	1
6.66	d	2

2.2.2 Carbon-13 Spectrum

The carbon-13 spectra is presented in Figure 3.



Spectral assignments are listed in Table II.

Table II

^{13}C NMR Spectral Assignments for Heroin [71]

<u>Chemical Shift</u>	<u>Multiplicity</u>	<u>Carbon Number</u>
119.1	d	1
121.6	d	2
132.0	s	3

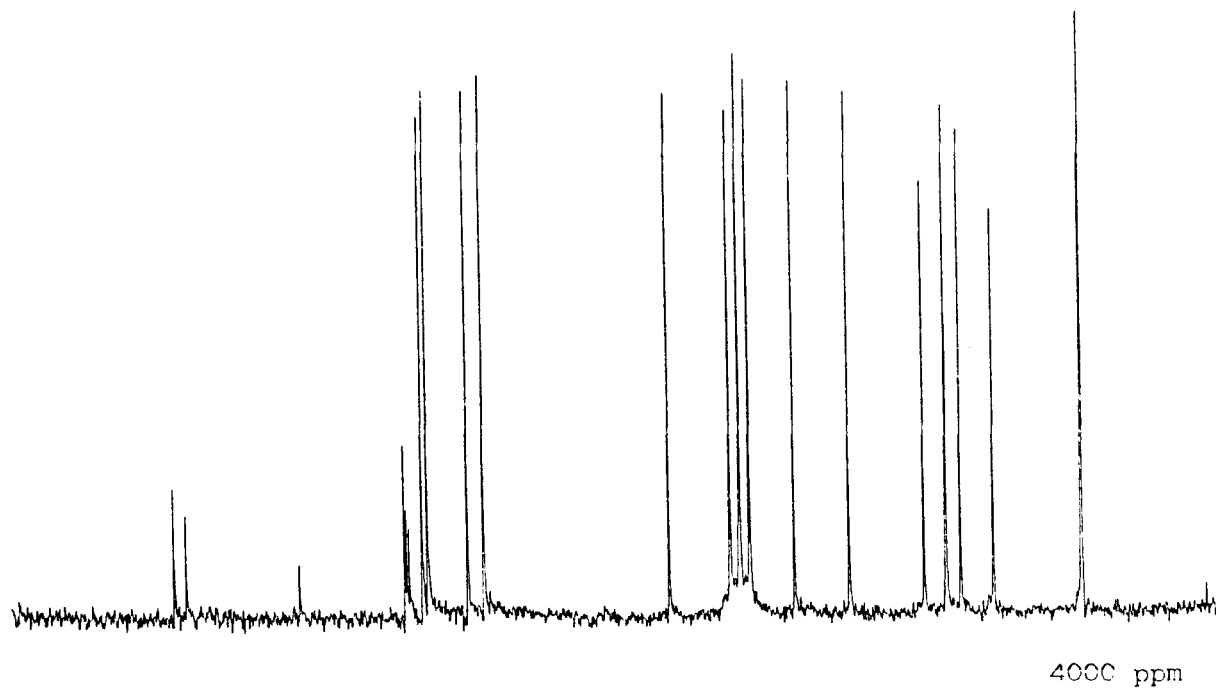


Figure 3a: carbon 13 NMR spectrum of heroin base

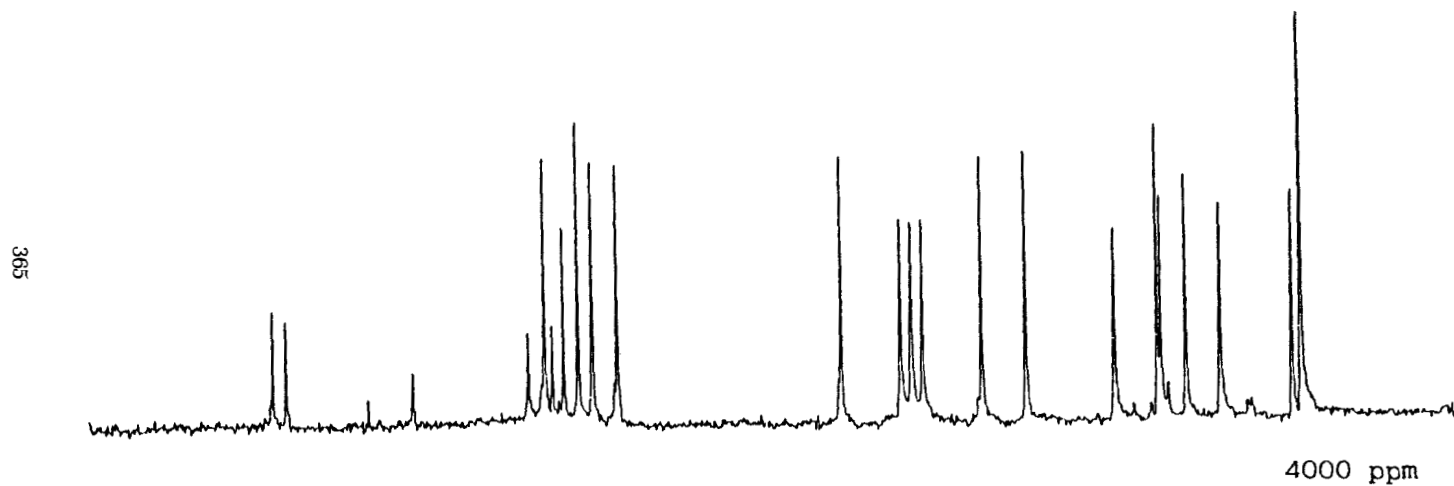


Figure 3b: carbon 13 NMR spectrum of heroin hydrochloride

Table II -- Cont'd.

<u>Chemical Shift</u>	<u>Multiplicity</u>	<u>Carbon Number</u>
149.1	s	4
88.5	d	5
67.9	d	6
129.2	d	7
128.2	d	8
58.7	d	9
20.4	t	10
131.5	s	11
131.2	s	12
42.6	s	13
40.4	d	14
34.9	t	15
46.3	t	16
42.8	q	NCH ₃
20.4	q	3CH ₃ CO
168.2	s	3CH ₃ CO
20.4	q	6CH ₃ CO
170.2	s	6CH ₃ CO

2.3 Ultraviolet spectra

The ultraviolet absorption spectra of heroin and heroin hydrochloride are shown in Figure 4 for the solvents listed in Table III (1 in 10,000 solutions used).

Table III

<u>Solvent</u>	<u>λ Max (nm)</u>	<u>Absorptivity</u>	
		<u>Heroin</u>	<u>Heroin HCl</u>
0.1 <u>N</u> hydrochloric acid	278	4.8 [5]; $E_{1\text{ cm}}^{1\%} = 39$ [4]	4.3 [53]
0.1 <u>N</u> sulfuric acid	279	4.8 [53]; $E_{1\text{ cm}}^{1\%} = 52$ [4]	4.3 [53]
ethanol	281	5.3 [53]; $E_{1\text{ cm}}^{1\%} = 54$ [68]	4.9 [53]

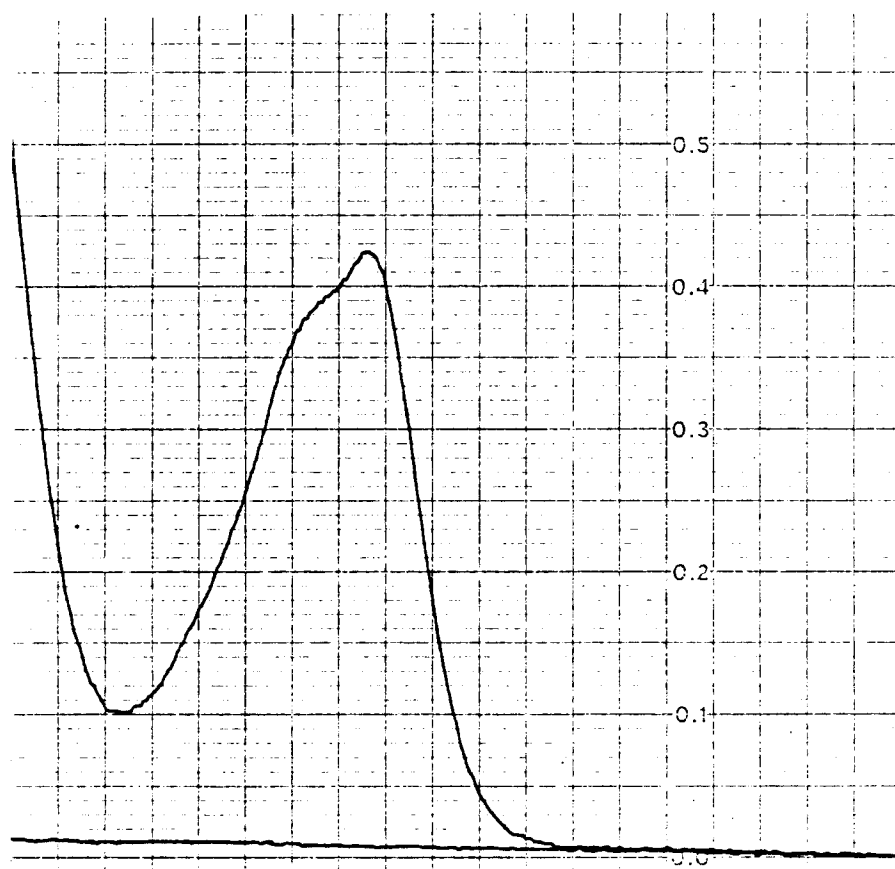


Fig. 4a. Ultraviolet spectrum in 0.1 N hydrochloric acid (representative of heroin or heroin hydrochloride).

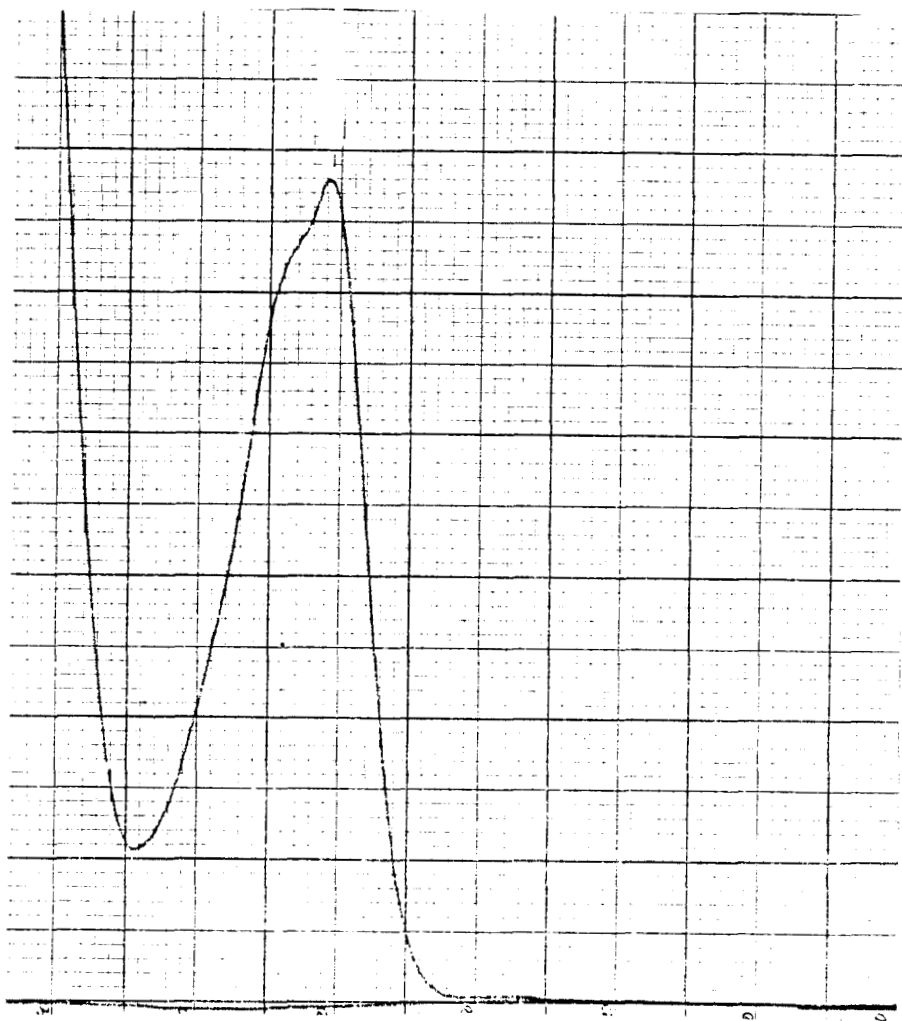


Fig. 4b. Ultraviolet spectrum in 0.1 N sulfuric acid
(representative of heroin or heroin
hydrochloride).

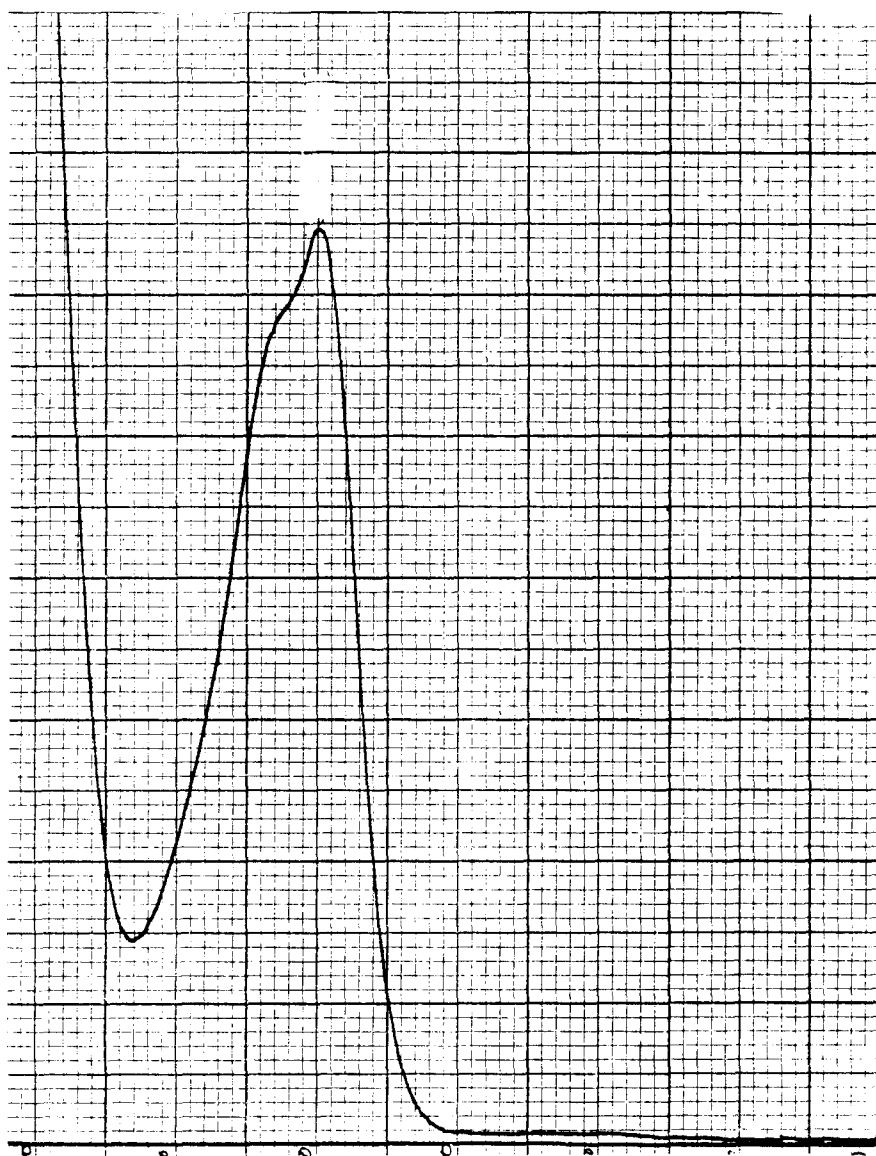


Fig. 4c. Ultraviolet spectrum in 95% ethanol (representative of heroin or heroin hydrochloride).

2.4 Mass Spectrum

The electron impact ionization spectrum is given in Figure 5, and the fragmentation pattern is presented in Table IV [6]. A Finnigan 3000 Peak Identifier mass spectrometer was used. The mass/charge (m/e) range scanned was 40 to 400 atomic mass units. The ionization potential was 70 eV.

Table IV

Mass Spectrum Fragmentation Pattern of Heroin [47, 54]

<u>m/e</u>	<u>Species</u>
369	M+
327	COCH ₂
268	M+ - CH ₃ COO and COCH ₂
310	M+ - CH ₃ COO (C ₆ acetyl group)
215	{ring cleavage followed by loss of peripheral groups
204	

2.5 Melting Range

The melting point of a heroin sample is about 170° [4]. It is also given as 173°C [2]. Additional melting ranges of 170°-172°C, 171°-174°C, and 172°-173°C and a melting point of 173°C have also been reported [24]. (See 2.11.)

The melting range of a heroin hydrochloride sample is between 229° and 233° [51,4]. It is also described as 243-244° [5].

2.6 Differential Scanning Calorimetry (DSC)

The DSC of heroin and heroin hydrochloride are shown in Figure 6 [53].

2.7 Solubility

The approximate solubilities obtained at room temperature are listed in Table V.

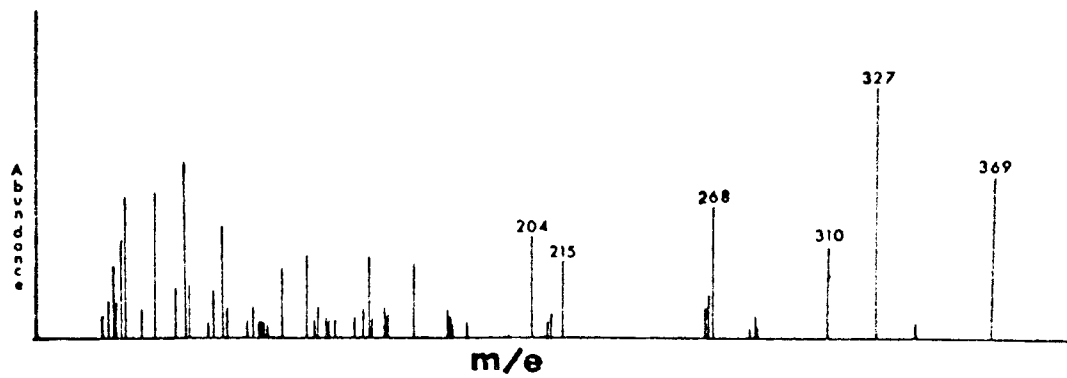


Figure 5: Mass Spectrum of Heroin

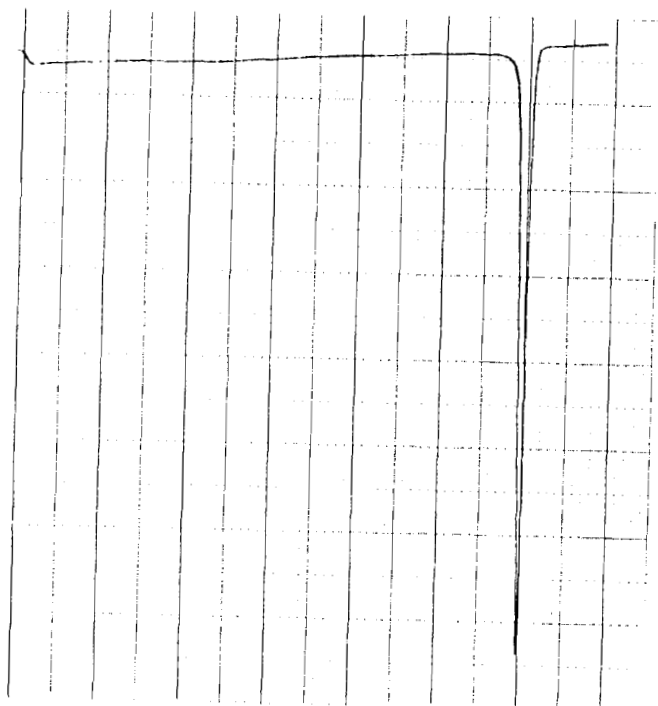


Fig. 6a. Differential scanning calorimetry of heroin base heating rate: 5/min.; 25 to 200 C.

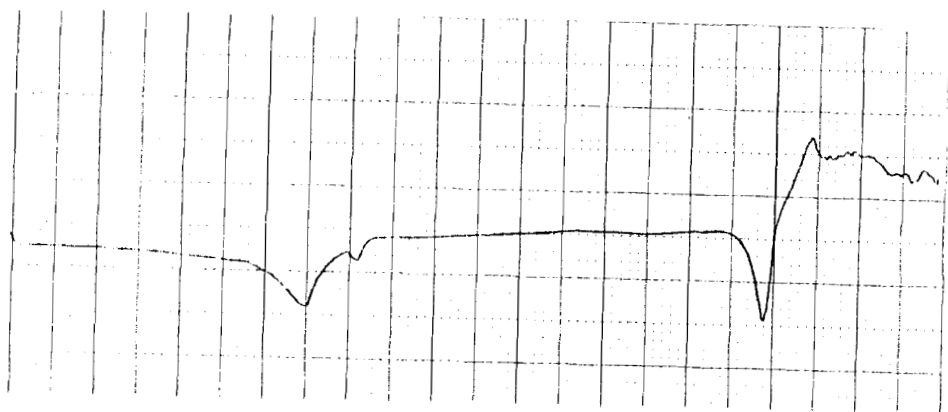


Fig. 6b. Differential scanning calorimetry of heroin hydrochloride heating rate: 5/min.; 25 to 300 C.

Table VSolubility Data of Heroin at Room Temperature

<u>Solvent</u>	<u>Approximate Solubility</u> (g/ml)	
	<u>Heroin</u>	<u>Heroin Hydrochloride</u>
water	1 in 1700 [2,4]	1 in 1.6 [51,4]; 1 in 2 [52]
ethanol	1 in 31 [2,4]	1 in 12 [51,4]; 1 in 11 [52]
ether	1 in 100 [2,4]	insoluble [51,4,52]
chloroform	1 in 1.5 [2,4]	1 in 1.6 [51,4]
alkali	soluble [2]	

2.8 Moisture Content2.8.1 Karl Fischer Titration

An accurately weighed sample of heroin or heroin hydrochloride is dissolved in methanol which has been titrated to end-point and titrated with Karl Fischer reagent using the dead stop end-point technique and a 20-second delay (heroin hydrochloride exists as the monohydrate) [53].

2.8.2 Loss on Drying

Heroin has been dried at 105° to constant weight [5].

Heroin hydrochloride has been dried at 105° to constant weight [51].

2.9 Specific Rotation

The specific rotation of heroin determined in 0.015 N methanolic hydrochloric acid at a concentration of 0.5% at 25°C is given in Table VI [5,53].

Table VISpecific Rotation data $[\alpha]^{25^\circ}$

<u>Wavelength (nm)</u>	<u>Specific Rotation (°)</u>	
	<u>Heroin [5]</u>	<u>Heroin Hydrochloride [53]</u>
589	-147	-133
578	-154	-139
546	-175	-159
436	-303	-275

The specific rotation of heroin in methanol $[\alpha]_D^{25}$ is -166° ($C = 1.49$) [2].

The specific rotation of heroin hydrochloride in water at 24° $[\alpha]_D^{25}$ is -156° ($C = 1.044$) [2].

2.10 Crystal Properties

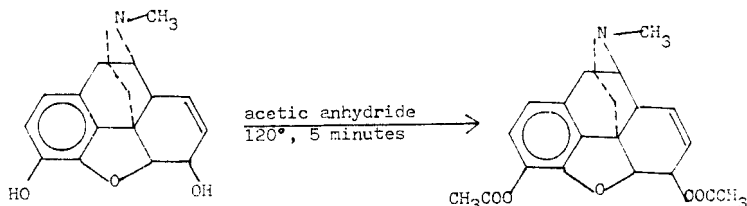
The crystal structure, configuration and bond distances are presented in Figures 7, 8, and 9. Heroin crystals were formed after addition of heroin hydrochloride to aqueous sodium acetate solution. Clear hexagonal crystals of diacetylmorphine free base were obtained. Reflections were measured with a Syntex P2 diffractometer with a 0-20 technique on a crystal $0.7 \times 0.2 \times 0.2$ mm [49].

2.11 Polymorphism

Heroin can exist in two polymorphic forms. Form I, consisting of rods, oblique plates, and needles, has a melting point of 172° - 173°C . Form II, consisting of spherulites, melts at about 168°C and is readily converted into Form I [24].

3. Synthesis

1. Synthesis from morphine [5].



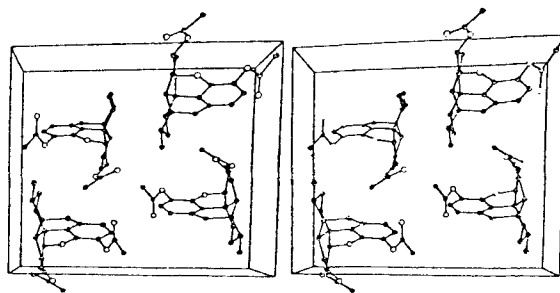


Fig. 7. Arrangement of the molecules in the unit cell (O oxygen). From an origin in the lower left front corner, c is to the right, b is vertical and a is into the page.

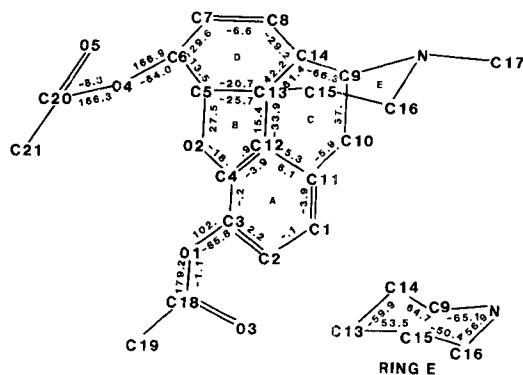


Fig. 8. Configuration drawing of diacetylmorphine with endocyclic torsion angles for rings A, B, C and D.

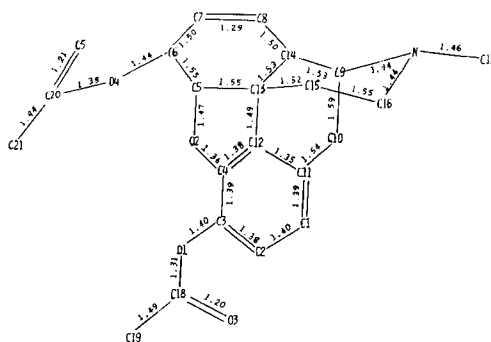
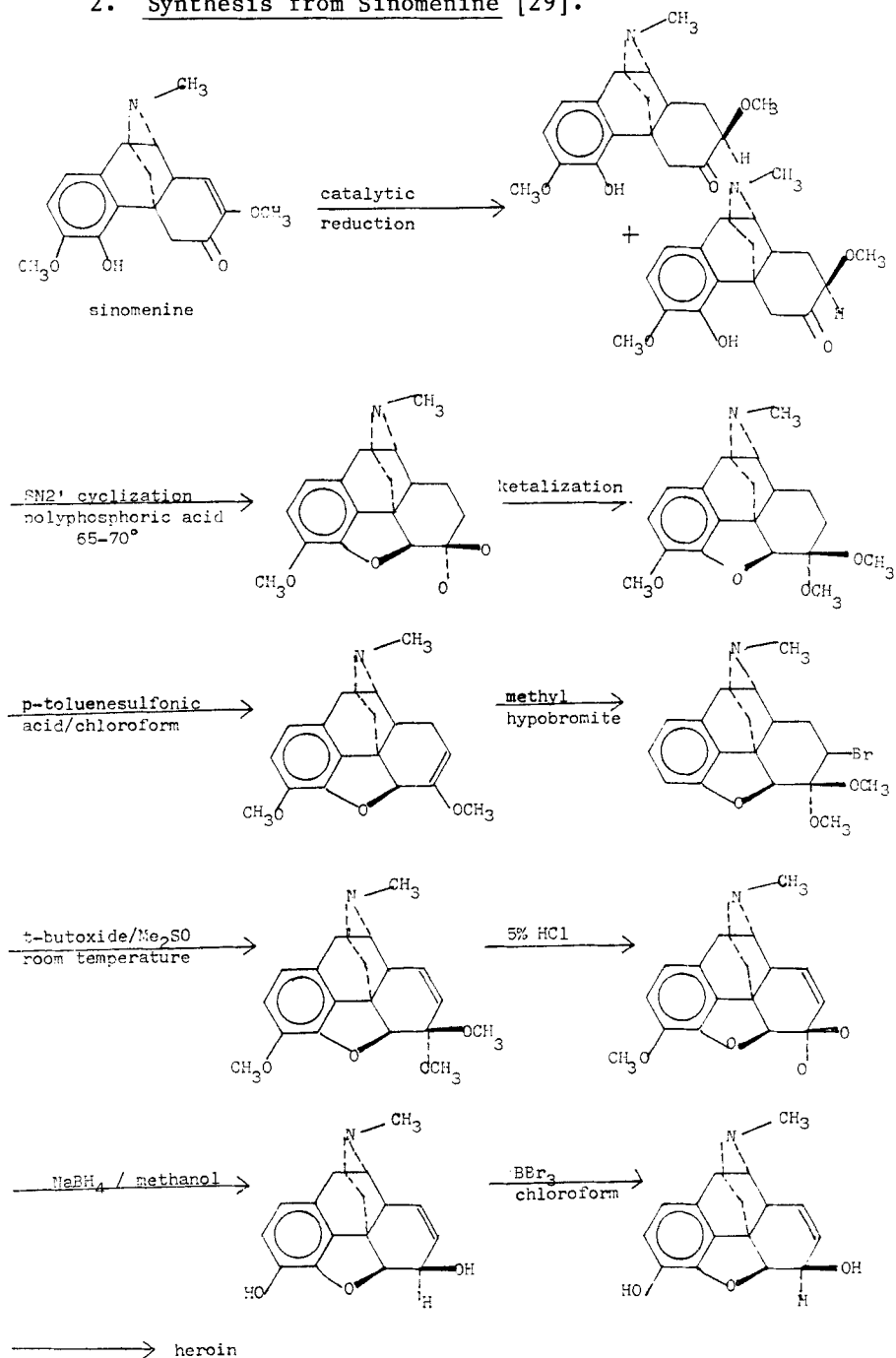


Fig. 9. Bond distances (A). The estimated average standard deviation in bond length is 0.014 A in diacetylmorphine.

2. Synthesis from Sinomenine [29].



4. Stability - Degradation

Heroin is rapidly hydrolyzed in alkaline solutions. It is rapidly hydrolyzed in vivo after mixing with blood to O^6 -monoacetylmorphine and then at a slower rate to morphine. Heroin also degrades to O^6 -monoacetylmorphine in buffered aqueous solutions (pH 7.4) at 23°C. The hydrolysis is more rapid at higher pH value (pH 6.4). No evidence of further conversion to morphine at pH 7.4 is observed in 24 hours [7]. Heroin hydrolyzes to O^6 -acetylmorphine in 0.5 M sodium carbonate with a half-life of only 4.2 min. Subsequent hydrolysis to morphine has a half-life of 55.5 min. [9]. Half-life for hydrolysis in human blood is 12.6 min.; in serum, 19.8 min. [9]. In pH 4 phosphate buffer, the half-life was 415 min.; in fresh dog plasma, 8 min. [9]. Heroin stability increases with increased alcohol content in Brompton mixtures [28]. Heroin reportedly is most stable at pH 4.0-4.5 [28] and at pH 4.3 [39].

5. Metabolism

Heroin is a short-acting (2 hours) narcotic analgesic. It is rapidly hydrolyzed in vivo by serum cholinesterase [38] to O^6 -monoacetylmorphine and then at a slower rate to morphine [4]. Heroin rapidly passes out of the blood [32] after conversion to O^6 -monoacetylmorphine and appears in the brain as O^6 -monoacetylmorphine where it is slowly hydrolyzed to morphine. Heroin and O^6 -monoacetylmorphine have a considerably greater ability to penetrate the blood brain barrier than does morphine which is the probable explanation for the higher potency of heroin [38]. O^6 -monoacetylmorphine, morphine, and morphine 3-glucuronide are the major metabolites of heroin excreted in the urine [9]. Minor or negligible amounts of normorphine and its glucuronide as well as morphine 6-glucuronide have been determined in urine [9]; dihydromorphinone [26], 6-acetylmorphine 3-glucuronide [26], and norcodeine [17] have also been detected in urine. Additional possible metabolic pathways of opiates in man are presented in Figure 10 [17].

6. Pharmacokinetics

Heroin is rapidly hydrolyzed to morphine and other metabolites and is rapidly excreted. Heroin, 6-acetylmorphine, morphine, the sum of morphine and 6-acetylmorphine and total normorphine, determined 24 hours after initial IV administration of a 10 mg/70 kg dose, was found to be 0.5, 1.5, 7.2, 54 and 4%, respectively, of administered dose.

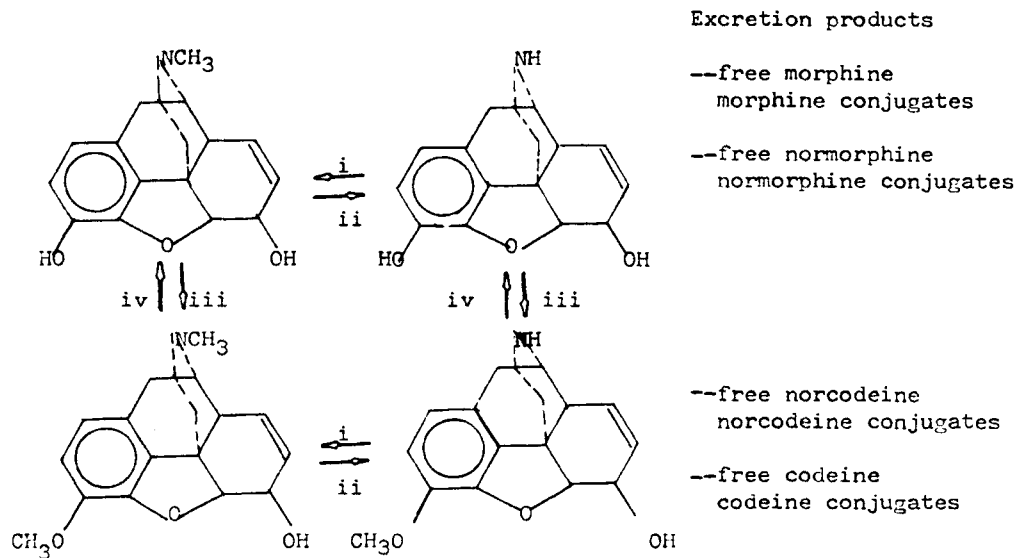


Figure 10: Possible metabolic pathways of opiates in man(i=N-methylation; ii=N-demethylation; iii= O-methylation; iv= O-demethylation)

Eighty-eight percent of free morphine and 84% of the total morphine (including morphine glucuronide) found in urine was excreted within the first eight hours [47]. Of the morphine found in the urine, 88% was bound as the glucuronide and 11% was free morphine [62] (50-60% bound and 7% free [26]). The amount of heroin detected in the urine as morphine after a single intramuscular injection of 5 mg is about one third that detected after a single intramuscular injection of 15 mg of morphine.

7. Methods of Analysis

7.1 Elemental Analysis

<u>Heroin</u>	<u>Heroin Theoretical % [2]</u>	<u>Heroin HCl (anhydrous) Theoretical % [2]</u>
carbon	68.28	62.14
hydrogen	6.28	5.96
nitrogen	3.79	3.45
oxygen	21.66	19.71
chlorine	----	8.74

7.2 Color tests

<u>Agent</u>	<u>Color</u>	<u>Ref.</u>
1. sulfuric acid-formaldehyde (Marquis)	purple (sensitivity 0.05 µg)	4,6
2. ammonium molybdate	red-purple-blue-light green (sensitivity 0.05 µg)	4
3. ammonium vanadate	faint blue-gray (sensitivity 1.0 µg)	4
4. Vitali's test	faint yellow & faint yellow/orange (sensitivity 1.0 µg)	4
5. nitric acid base (heroin HCl)	yellow green	20,51
6. Mecke test	deep green	6
7. cobalt thiocyanate	purple	42

<u>Agent</u>	<u>Color</u>	<u>Ref.</u>
8. sulfuric acid- potassium hexacy- anoferrate (III)- iron (III) chloride heroin HCl)	deep blue	51
9. nitric acid- phosphoric acid	yellow to red brown (depending on concentration)	57

7.3 Microcrystalline Tests

<u>Agent</u>	<u>Crystal type</u>	<u>Ref.</u>
mercuric iodide	needles	20
sodium acetate	hexagon	20
platinum chloride	needles	6,20
gold bromide	needles	6,20
mercuric chloride- hydrochloric acid	blades and needles	6,20
iodine-potassium iodide	blades and needles	70
bromauric acid-phosphoric acid-hydrogen bromide	amorphous precipitate followed by irregular dichroic plates or blades	70

7.4 Non-aqueous Titrimetric Analysis

An accurately weighed sample of heroin is dissolved in glacial acetic acid and titrated to the potentiometric end-point with 0.1 N acetous perchloric acid, using a glass indicating electrode and a calomel reference electrode filled with 0.02 N lithium chloride in glacial acetic acid. The titrant is standardized against dried potassium biphthalate. A blank titration is run [5].

An accurately weighed sample of heroin hydrochloride is dissolved in glacial acetic acid; mercuric acetate is added. The solution is titrated to the potentiometric end-point with 0.1 N acetous perchloric acid using a glass indicating electrode and a calomel reference electrode filled with 0.02 N lithium chloride in glacial acetic acid. The titrant is standardized against dried potassium biphthalate. A blank titration is run [53]. Crystal violet indicator may also be used [51].

7.5 Chloride Titration

An accurately weighed sample of heroin hydrochloride is dissolved in 1.5 N sulfuric acid and titrated to the potentiometric end-point with 0.1 N silver nitrate using a silver indicating electrode and a mercurous sulfate reference electrode. The titrant is standardized with dried sodium chloride and a blank titration is run [53].

7.6 Phase Solubility Analysis

The United States Pharmacopeia procedure was followed [52]. The heroin solvent was (3:1) hexane:dioxane (solubility 13 mg/g) [5]. Dioxane was used for heroin hydrochloride (solubility 26 mg/g) [53]. The solvents were commercial distilled-in-glass solvents which had been degassed prior to mixing. The bath temperature was 25°, rotation was 28 rpm.

7.7 Thin-layer Chromatography

Thin-layer chromatography has frequently been used for the analysis of heroin. Methods of detection and solvent systems are listed in Table VII.

7.8 Paper Chromatography

Ascending paper chromatography was accomplished using Whatman #1 paper which had been buffered by dipping into a 5% solution of sodium dihydrogen citrate, blotting, and drying at 25° for 1 hour. The solvent consisted of 4.8 g of citric acid in a mixture of 130 ml of water and 870 ml of 1-butanol. 2.5 μ l of a 1% solution in 2 N acetic acid, 2 N hydrochloric acid, 2 N sodium hydroxide, or ethanol were spotted on the paper. Visualization was accomplished using ultraviolet light or iodoplatinate spray (R_f = 0.33) [4].

Reversed phase ascending paper chromatography was conducted using Whatman #1 or #3 paper impregnated by dipping into a 10% solution of tributyrin in acetone and drying in air. Acetate buffer pH 4.58 was used as solvent. Samples were spotted from a 1 to 5% solution in ethanol or chloroform. Iodoplatinate spray was used for detection (R_f = 0.84) [4].

An additional reversed phase ascending paper chromatography system consisting of phosphate buffer (pH 7.4) solvent and Whatman #1 or #3 paper impregnated with a

Table VII

Thin-layer Chromatography of Heroin

<u>Plate</u>	<u>Solvent</u>	<u>Method of Detection</u>	<u>Rf x 100</u>	<u>Ref.</u>
silica gel	methanol:aqueous ammonia (100:1.5)	A,B,C,D,E,J	45, 38, 50	4,5,20,56 ---
silica gel	aqueous ammonia-benzene- dioxane-ethanol (5:50:40:5)	B	76, 60	4, 20
silica gel	acetic acid-ethanol-water (30:60:10)	A,B,C,D	35,44,50, 35,35	4,5,20,21, 22
382 silica gel F-254	benzene-dioxane-ethanol- 25% aqueous ammonia (10:8:1:1)	A,B,C,D	46,76	5,21
silica gel F-254	methanol	A,B,C,D	38	5
cellulose	2-propanol-water-glacial acetic acid (8:1:1)	A,B,C,D	72	5
silica gel	butyl ether-ethyl ether- diethylamine (45:45:10)	A	44	6
silica gel	chloroform-dioxane-ethyl acetate-aqueous ammonia (25:60:10:5)	A	85	6
silica gel	chloroform-methanol (9:1)	A,C,J	61,--	6,56

Table VII -- Cont'd.

<u>Plate</u>	<u>Solvent</u>	<u>Method of Detection</u>	<u>Rf x 100</u>	<u>Ref.</u>
silica gel	chloroform saturated with ammonia-methanol (18:1)	A	70	6
silica gel	chloroform-methanol (8:2)	E	77,--	9,67
silica gel	chloroform-cyclohexane-diethylamine (8:10:3)	A,E	-----	18
HPTLC-silica gel F-254	toluene-methanol-aqueous ammonia (50:50:1)	A,F	60	19
HPTLC-silica gel F-254	2-propanol-n-heptane-aqueous ammonia (50:50:1)	A,F	10	19
silica gel G+ 0.1 M KOH	cyclohexane-benzene-diethylamine (75:15:10)	-----	22	21
silica gel G+ 0.1 M KOH	methanol	-----	39	21
silica gel G+ 0.1 M KOH	acetone	-----	20	21
silica gel+ 0.1 M NaHSO ₄	methanol	-----	24	21
silica gel+ 0.1 M NaHSO ₄	95% ethanol	-----	9	21

Table VII -- Cont'd.

<u>Plate</u>	<u>Solvent</u>	<u>Method of Detection</u>	<u>Rf x 100</u>	<u>Ref.</u>
silica gel	methanol- <u>n</u> -butanol-benzene- water (60:15:10:15)	E	35,35	21,22
silica gel	ethanol-pyridine-dioxane- water (50:20:25:5)	E	37,37	21,22
cellulose (previously dipped in 5% sodium dihydro- gen citrate and dried one hour)	citric acid (4.8 g) in water- <u>n</u> -butanol (130:870)	A,E,K	43	61
silica gel	alcohol- <u>n</u> -butyl ether- water (80:7:13)	E	15,15	21,22
silica gel	<u>n</u> -butanol-glacial acetic acid-water (4:1:2)	E	61,61	21,22
silica gel	<u>n</u> -butanol-concentrated HCl saturated with water (9:1)	E	32,32	21,22
MN-cellulose powder 300 G	methanol- <u>n</u> -butanol-benzene- water (60:15:10:15)	E	90,90	21,22
MN-cellulose powder 300 G	<u>t</u> -amyl alcohol- <u>n</u> -butyl- ether-water (80:7:13)	E	95,65	21,22

Table VII -- Cont'd.

<u>Plate</u>	<u>Solvent</u>	<u>Method of Detection</u>	<u>Rf x 100</u>	<u>Ref.</u>
silica gel	chloroform-dioxane-ethyl- acetate-aqueous ammonia (25:60:10:5)	E	67	21
silica gel	ethanol-chloroform-dioxane- petroleum ether-benzene- aqueous ammonia-ethyl acetate (5:10:50:15:10:5:5)	E	73	21
silica gel	ethyl acetate-benzene-aqueous ammonia (60:35:5)	E	19	21
silica gel	ethanol-dioxane-benzene- aqueous ammonia (5:40:50:5)	-----	76	22
silica gel	acetone-methanol-aqueous ammonia (50:50:1)	-----	58	23
silica gel	chloroform-acetone-aqueous ammonia (50:50:1)	-----	64	23
silica gel	ethanol-dioxane-benzene- aqueous ammonia (5:40:50:5)	-----	76	23
silica gel	ethanol-di-n-butyl ether- aqueous ammonia (60:35:5)	-----	11	23

Table VII -- Cont'd.

<u>Plate</u>	<u>Solvent</u>	<u>Method of Detection</u>	<u>Rf x 100</u>	<u>Ref.</u>
silica gel	chloroform-acetone (9:1)	A,C,J	--	56

Method of Detection

- A. shortwave ultraviolet light
- B. longwave ultraviolet light
- C. 0.5% iodine in chloroform
- D. acidified iodoplatinate followed by exposure to ammonia vapor
- E. potassium iodoplatinate
- F. Dragendorff's reagent followed by heat (120°C) for 5 minutes and spraying with 10% sulfuric acid.
- G. potassium permanganate
- H. cobalt thiocyanate
- I. bromocresol green
- J. iodine in methanol + copper chloride (Ludy-Tenger)
- K. dilute hydrochloric acid

10% solution of tributyrin in acetone was used. The samples were spotted using a 1 to 5% solution in ethanol or chloroform. Iodoplatinate spray detection was used ($R_f = 0.12$) [4].

Paper chromatography was also conducted using the systems in Table VIII.

Table VIII
Paper Chromatography of Heroin*

<u>Paper + Treatment</u>	<u>Solvent</u>	<u>Detection</u>	<u>R_fx100</u>	<u>Ref.</u>
Whatman #1 impreg- nated with formalde- hyde and 1% acetic acid	chloroform	-----	76	21
Whatman #1 impreg- nated with 5% zir- conium phosphate	5% acetic acid	-----	33	21
Whatman #1.	1-butanol- glacial acetic acid-water (12:3:5)	C,E,G,H,I	74	21
Whatman #1.	1-butanol-1 <u>N</u> sodium acetate- 1 <u>N</u> hydrochloric acid (7:120:60)	C,E,G,H,I	89	21
Whatman #1 buffered with 5% sodium di- hydrogen citrate	1-butanol- glacial acetic acid-water (12:3:5)	C,E,G,H,I	32	21
Whatman #1 buffered with 5% sodium di- hydrogen citrate	1-butanol-1 <u>N</u> sodium acetate- 1 <u>N</u> hydrochloric acid (7:120:60)	C,E,G,H,I	16	21

* Spotting solvent was not given.

7.9 Gas Chromatography

Gas chromatographic systems used for analysis are listed in Table IX. Flame ionization detection was used unless otherwise specified.

7.10 High-Performance Liquid Chromatographic Analysis

High-Performance Liquid Chromatography has been used extensively for the analysis of heroin. The various HPLC systems used for the analysis are given in Table X.

8. Determination in Biological Fluids

Plasma-Serum

Organic solvent extracts were obtained with ethyl acetate-isopropanol (85:15), or benzene-butanol (85:15) from plasma mixed with pH 8.9 carbonate buffer. The extracts were dried and reconstituted in acetone and analyzed on a bonded phase column using a mobile phase consisting of methanol (0.1% $(\text{NH}_4)_2\text{CO}_3$ and 0.01 M $(\text{NH}_4)_2\text{HPO}_4$, pH 6.98) (55:45) and UV detection (254 nm). Heroin, 6-O-acetylmorphine, and morphine were separated [9]. Similarly, TLC was used for separation of heroin, morphine, 6-O-acetylmorphine and morphine glucuronide [9] on silica gel plates using chloroform-methanol (80:20) and iodoplatinate for visualization.

Blood

Heroin has been analyzed by gas chromatography following the extraction scheme presented for urine below [62]. Silyl derivatives was analyzed on QF-1 or SE-30.

Urine

The following procedure was used for the quantitation of heroin and morphine by gas chromatography. Extraction from sodium bicarbonate solution into ethyl acetate is followed by extraction with 0.05 N hydrochloric acid. Ammonium hydroxide, sodium chloride, and sodium bicarbonate are added to the hydrochloric acid solution. Extraction with ethyl acetate, silylation and analysis on QF-1 and SE-30 using flame ionization detection follows [62]. Metabolites in urine were also determined after acid hydrolysis and silylation followed by gas chromatography [26]. An alternative analysis using thin-layer

Table IX
Gas Chromatography of Heroin

<u>Column</u>	<u>Support</u>	<u>Mesh</u>	<u>Length</u>	<u>Temp. (°)</u>	<u>Flow (ml/min); Carrier Gas</u>	<u>Ref.</u>
1% SE-30	Anakrom ABS	100-120	6 ft x 4 mm glass	250	80; argon	4
2.5% SE-30	Chromosorb WAW HMDS	80-100	5 ft x 4 mm glass	225	50; nitrogen	4
5% SE-30	Chromosorb WAW	60-80	5 ft x 1/8" stainless steel	230	30; nitrogen	4,21
3% OV-1	Gas Chrom Q	100-120	1.2 m x 4 mm glass	210	50; helium	5
3% OV-17	-----	----	6 ft x 4 mm glass	250	-----	6
3% OV-1	-----	----	6 ft x 4 mm glass	250	-----	6
10% OV-1*	Chromosorb GHP	----	0.6 m x 4 mm glass	220	40; 5% methane in argon	7
3% OV-17*	Gas Chrom Q	100-120	1.83 m x 4 mm glass	220-240	100-120; nitrogen	8,14

Table IX -- Cont'd.

<u>Column</u>	<u>Support</u>	<u>Mesh</u>	<u>Length</u>	<u>Temp. (°)</u>	<u>Flow (ml/min); Carrier Gas</u>	<u>Ref.</u>
0.04% SDBS 0.02% FFAP 0.06% SP-525	glass beads	70-90	1.6 m x 3 mm glass	240	0.5 kg/cm ² ; nitrogen	13
3% OV-17	Gas Chrom Q	80-100	1 m x 6.35 mm o.d. glass	235	55; nitrogen	16
3% OV-17	Chromosorb WHP	-----	-----	260	-----	18
3% OV-1	Chromosorb W	80-100	1.2 m x 6.35 mm o.d. glass	250	60; nitrogen	20
3% OV-17	Chromosorb W	80-100	1.2 m x 6.35 mm o.d. glass	280	60; nitrogen	20
5% SP-2401-DB	Supelcoport	100-120	1.2 m x 2 mm i.d. glass	250	60; helium	55
5% SP-2401-DB	Supelcoport	100-120	1.2 m x 2 mm i.d. glass	255	50; helium	55
3% SP-2401-DB	Supelcoport	100-120	1.2 m x 2 mm i.d. glass	230, 245	--- -----	55

Table IX -- Cont'd.

<u>Column</u>	<u>Support</u>	<u>Mesh</u>	<u>Length</u>	<u>Temp. (°)</u>	<u>Flow (ml/min); Carrier Gas</u>	<u>Ref.</u>
3% OV-25	Gas Chrom Q	80-100	1.8 m x 3.18 mm stainless steel	240	30; nitrogen	20
6% Dexsil 400	Gas Chrom Q	80-100	1.8 m x 3.18 mm stainless steel	240	30; nitrogen	20
1% SE-30	Chromosorb W	-----	1.83 m x 4 mm glass	210, 225, 250	(argon β -ioniza- tion)	21
0.1% poly- ethylene glycol 9000 + 1.15% SE-30	Chromosorb P washed with concentrated HCl and methanolic potassium hydroxide and treated with hexamethyldisilazane	-----	1.83 m x 3 mm glass	175, 200, 225	(argon β -ioniza- tion)	21
1% Hi EFF- 8B	Gas Chrom P	100-120	0.92 m x 3.2 glass	220, 250	HFI	21
3% cyclohex- ane dimethanol succinate	Chromosorb W	80-100	5 ft x 4 mm i.d.	200, 250	60; nitrogen	61

Table IX -- Cont'd.

<u>Column</u>	<u>Support</u>	<u>Mesh</u>	<u>Length</u>	<u>Temp. (°)</u>	<u>Flow (ml/min); Carrier Gas</u>	<u>Ref.</u>
3% SE-30**	Chromosorb WHP	100-120	5.3 ft x 2 mm i.d.	218	95% of controller; 62 nitrogen	
2.7% QF-1	Chromosorb WHP	100-120	9 ft x 2 mm i.d.	218	60% of controller; 62 nitrogen	
3% OV-17- 5% SE-30 (1:1)	Varoport 30 (OV-17) Chromosorb WAW (SE-30)	80-100	6 ft x 2 mm i.d.	temperature program: 250 (12 min); 10/ min, 280 (12 min)	30 ml/min; helium	65
3.8% UCW-98	Chromosorb WHP	80-100	6 ft x 4 mm	225	40 ml/min; helium	67

*Derivatized with heptafluorobutyric acid anhydride in acetonitrile, 5 min., 60°C.

**Derivatized with BSA.

Table X

High-Performance Liquid Chromatographic Systems for Heroin

<u>Column</u>	<u>Mobile Phase</u>	<u>Temperature</u>	<u>Flow/ Pressure</u>	<u>Detector (λ nm)</u>	<u>Ref.</u>
SCX (1.0 m)	0.4-1.4 M sodium perchlorate in 0.01 M pH 6.8 aqueous phosphate buffer containing 10% ethanol	35°	1000 psi	UV (254)	5
bonded phase	methanol-(0.1% ammonium carbonate 0.01 M (NH ₄) ₂ HPO ₄) pH 6.98 (6:4); (55:45)	ambient	2 ml/min	UV (—)	9
LiChrosorb Si60 (10 μm)	0.2 N aqueous ammonia	ambient	3 ml/min	-----	12
μBondapak C ₁₈	acetonitrile-(aqueous buffer containing 0.75 g ammonium acetate) (65:35)	ambient	1.5 ml/min	UV (280)	15
LiChrosorb Li 60 (5μ)	diethyl ether-iso-octane-methanol-diethylamine (52.8:35:12:0.2)	ambient	1.5 ml/min 175 bar pressure	UV (250)	18

Table X -- Cont'd.

<u>Column</u>	<u>Mobile Phase</u>	<u>Temperature</u>	<u>Flow/ Pressure</u>	<u>Detector (λ nm)</u>	<u>Ref.</u>
μ Bondapak C ₁₈	acetonitrile-(0.015 M monobasic potassium phosphate adjusted to pH 3.0 with 2 N phosphoric acid) (1:3)	ambient	0.8 ml/min (620 psi)	UV (235)	28
μ Bondapak C ₁₈ (heroin hydrochloride)	acetonitrile-(0.015 M KH ₂ PO ₄ adjusted to pH 3.5 with 2 N phosphoric acid) (3:7)	ambient	1 ml/min (800 psi)	UV (235)	39
Whatman Partisil-10 ODS (heroin hydrochloride)	acetonitrile-water with 0.1% (NH ₄) ₂ CO ₃ (6:4)	ambient	2 ml/min	UV (254)	40
μ Bondapak C ₁₈	(50% methanol/0.05 M phosphate buffer pH 6.2)-methanol; 0-100% methanol, 1%/min linear gradient	ambient	1.2 ml/min	UV (254)	54
μ Bondapak C ₁₈	(50% methanol/0.05 M phosphate buffer pH 7.4)-methanol; 0-100% methanol, 1%/min linear gradient	ambient	1.2 ml/min	UV (254)	54

Table X -- Cont'd.

<u>Column</u>	<u>Mobile Phase</u>	<u>Temperature</u>	<u>Flow/ Pressure</u>	<u>Detector (λ nm)</u>	<u>Ref.</u>
Zipax SCX	(0.2 M H_3BO_3 adjusted to pH 9.3 with 40% sodium hydroxide)-(0.2 M H_3BO_3 -acetonitrile-n-propanol (86:12:2) adjusted to pH 9.8 with 40% sodium hydroxide); 0-100% linear gradient	ambient	2 ml/min	UV (270)	60
Corasil II	hexane-(chloroform-methanol-diethylamine (100:300:1)) gradient	ambient	600 psi	UV (254)	64
Merckosorb Si-60	chloroform-methanol (9:1, 8:2, 7:3)	20°	50-250 kg/cm ²	UV (254)	68
Merckosorb Si-60	diethylether-methanol (8:2, 7:3, 6:4)	20°	50-250 kg/cm ²	UV (254)	68

chromatography with a 1-butanol-acetic acid-water (35:3:10) system on silica gel plates and ethyl acetate-methanol-ammonium hydroxide (17:2:1) on silica gel plates has also been reported [26]. Plates were visualized using iodoplatinate spray. Morphine metabolites in urine were also analyzed after incubation with acid followed by adjustment to basic pH and benzene extraction. Thin-layer chromatography was then done using ethanol-benzene-1,4-dioxane-concentrated aqueous ammonia (50:40:5:5) and 1,4-dioxane-chloroform-ethyl acetate-concentrated aqueous ammonia (60:25:10:5) systems, silica gel plates, and potassium iodoplatinate spray [17,66].

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HYDROCHLOROTHIAZIDE

Hans Peter Deppeler

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1. Description

1.1 General Information

Research in sulfonamide chemistry has brought a rich yield of valuable therapeutics. One of the great successes was the discovery of the benzothiadiazines as potent diuretics of low toxicity(1). In 1958 De Stevens et al.(2) reported on the condensation product of 4-amino-6-chloro-3,5-disulfonamide and formaldehyde which was found to be identical with the hydrogenation product of chlorothiazide(3) and which soon became a widely used saluretic: Hydrochlorothiazide

1.2 Nomenclature

1.2.1 Chemical Names

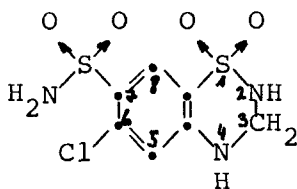
Hydrochlorothiazide is the recommended international nonproprietary name(4) of
6-Chloro-3,4-dihydro-7-sulfamoyl-2H-1,2,4-benzothiadiazine 1,1-dioxide(5) or
6-Chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide(5,6) or
6-Chloro-7-sulfamyl-3,4-dihydro-1,2,4-benzothiadiazine 1,1-dioxide(5) or
2H-1,2,4-Benzothiadiazine-7-sulfonamide, 6-chloro-3,4-dihydro-, 1,1-dioxide(6)
CAS registry number: 58-93-5

1.2.2 Trade Names

The Merck Index(5) quotes 28, and Index Nominum(7) 64 trade names not including the combinations with other active substances. Therefore, only a few examples can be listed here. Trade names including combinations in different countries:

France:	Adelphan-Esidrex, Esidrex, Esimil, Hydromet, Moduretic
Germany(BRD):	Di-Chlotride, Diu 25, Esidrix
Germany(DDR):	Disalunil, Urodiazin
Great Britain:	Direma, Esidrex, Hydrosaluric, Salupres
Japan:	Esidrix, Dichlotride
USA:	Esidrix, Hydrodiuril, Oretic, Serapes, Thiuretic.

1.3 Formula and Molecular Weight



(5,6)

$C_7H_8ClN_3O_4S_2$ Molecular Weight 297.73

1.4 Appearance

White, or practically white, practically odourless, crystalline powder(6). Slightly bitter taste(8).

1.5 Official Compendia

Monographs on hydrochlorothiazide and hydrochlorothiazide tablets are included in the following compendia:

BP 73, DAB 7 (DDR), Ph. Int. II, Ph. Jap. 1971, Ph. Nord. Add., USP XIX

Monographs in Ph. Eur. are proposed. A USP Hydrochlorothiazide Reference Standard is available.

1.6 Other Compendia

Summaries including analytical and pharmaceutical informations are given in The Pharmaceutical Codex(9) and in Kirk-Othmer, Encyclopedia of Chemical Technology(10).

2. Physical Properties

2.1 Spectra

2.1.1 Infrared(11)

The infrared spectrum is presented in Figure 1. The spectrum was obtained from a mineral oil mull on a Perkin Elmer Model 157 infrared spectrophotometer in the range of $4000-650\text{ cm}^{-1}$. Assignments for the characteristic bands in the spectrum are listed in Table I.

Table I

Infrared absorption

Wavenumber cm^{-1}	Assignments
3370, 3270, 3170 1600, 1550, 1520	NH + NH ₂ heterocyclic ring system
1335 / 1320 1180 / 1165 / 1150	SO ₂

2.1.2 Raman(11)

The Raman spectrum of hydrochlorothiazide powder is shown in Figure 2 and Table II. It was obtained on a Cary Model 83 spectrometer using the argon 488 nm excitation of a Lexel Model 75 ion laser

Table II

Raman spectrum

Frequency cm^{-1}	Assignments
3380, 3280, 3180 3080, 3020 2960, 2900 1600, 1525, 1460 1335, 1320 1165, 1155 940, 900 710, 675	NH stretching aromatic CH stretching CH ₂ stretching C=C stretching SO ₂ asym. stretching SO ₂ sym. stretching S-N stretching + NH deformation ring deformations

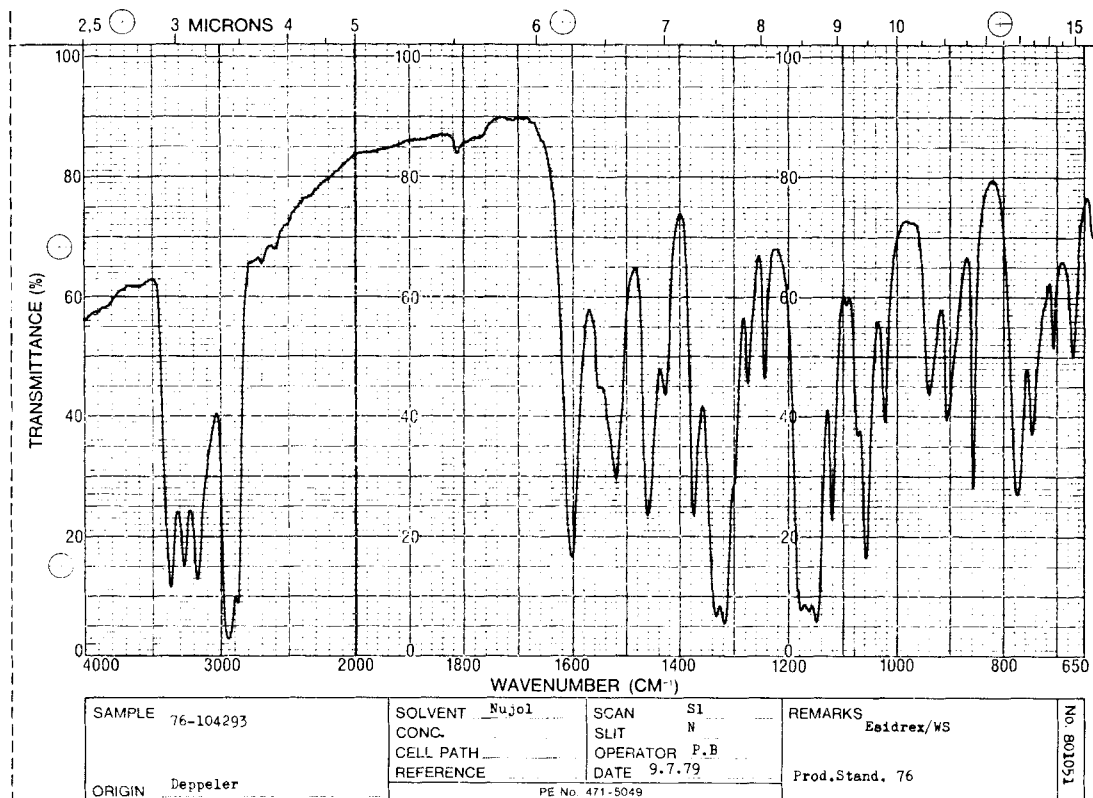


Fig. 1. Infrared spectrum of hydrochlorothiazide.

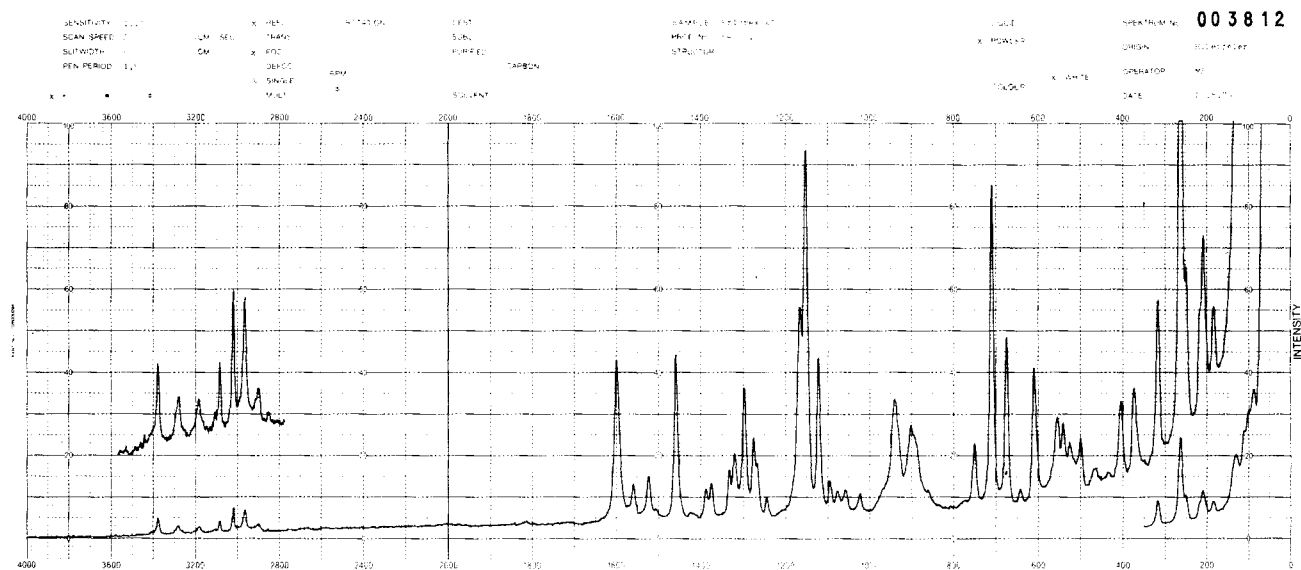


Fig. 2. Raman spectrum of hydrochlorothiazide.

2.1.3 Ultraviolet

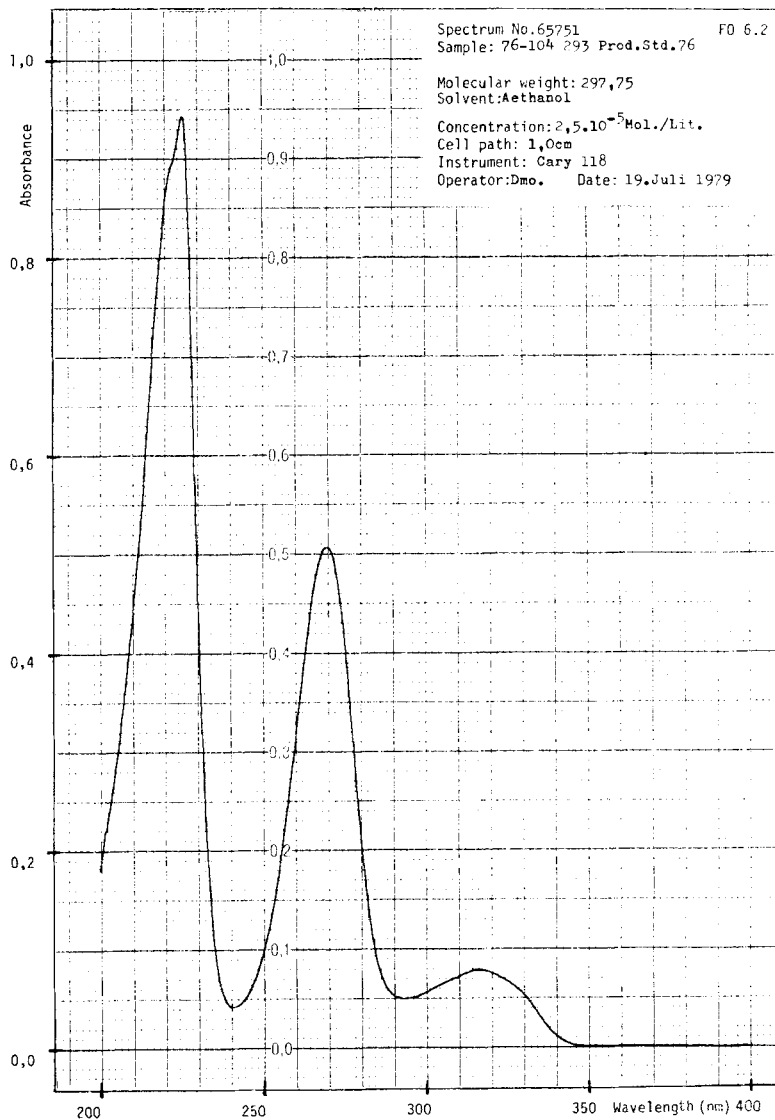
The UV spectrum of hydrochlorothiazide in ethanol is shown in Figure 3 (11). Information about the UV absorption in other solvents is given in Table III.

Table III

Ultraviolet absorption

Solvent	λ max nm	λ min nm	log ϵ
ethanol(11)	225		4.576
	269		4.307
	316		3.505
methanol(12)	226		4.513
		241.5	3.129
	271		4.279
		294	3.272
	317		3.471
water(13)	270		4.286
	315		3.495
	270		4.290
0.01 N HCl(13)	315		3.500
0.01 N NaOH(13)	272		4.193
	323		3.435
0.1 N NaOH(14)	221		4.448
		247	
	273		4.198
	319/320	299	3.456

Figure 3. Ultraviolet spectrum of hydrochloro-thiazide in ethanol



2.1.4 ^1H -Nuclear Magnetic Resonance(11)

The ^1H -NMR spectrum shown in Figure 4 was obtained from a solution in acetone- d_6 at ambient temperature on a Varian XL-100-12 spectrometer at 100 MHz. The assignments of the signals are listed in Table IV.

Table IV

 ^1H -NMR

Signal	Chem. shift ppm vs. TMS	Multiplicity	Number of protons	Species
a	8.16	singlet	1	arom. proton
b	7.16	broad singlet	1	sulfon- amide NH
c	7.04	singlet	1	arom. proton
d	6.75	broad singlet	1	arom. NH
e	6.65	broad singlet	2	Ar-SO ₂ NH ₂
f	4.93	broad singlet	2	methylene protons
g	3.0	singlet	-	solvent
h	2.05	multiplet	-	solvent

Signal f is broadened due to unresolved coupling with the NH protons.

NMR spectrum no. 105523

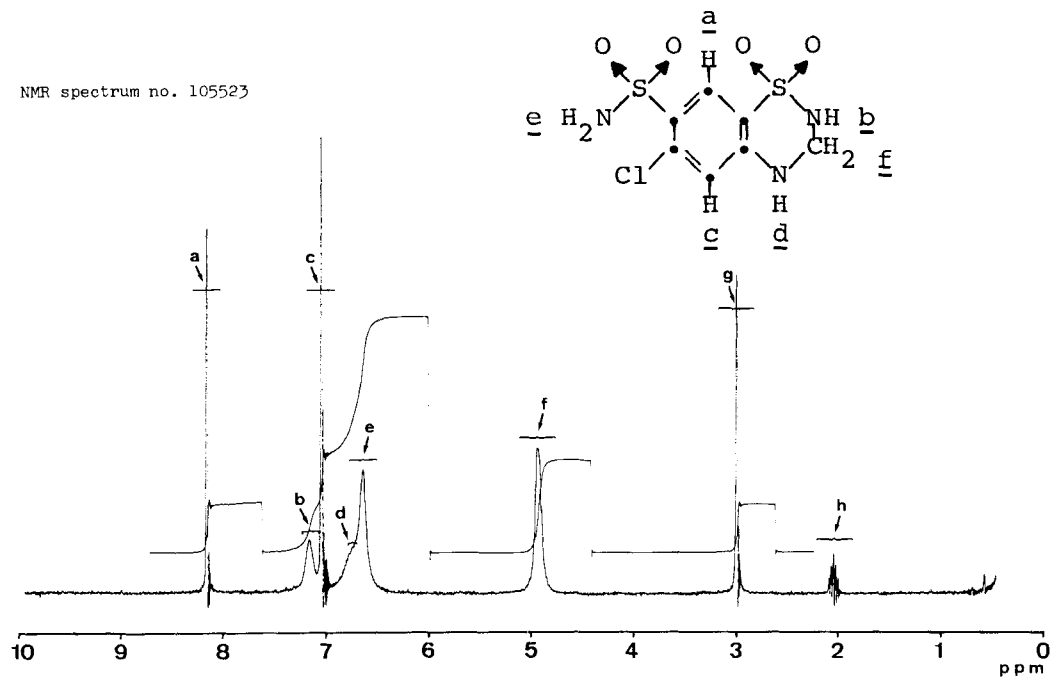


Figure 4. ^1H -NMR spectrum of hydrochlorothiazide

2.1.5 ^{13}C -Nuclear Magnetic Resonance(11)

The ^{13}C -NMR spectra shown in the Figures 5, 6, 7 were recorded at 25.2 MHz and ambient temperature with a Varian XL-100-15 spectrometer using a solution in acetone- d_6 . Figures 5 and 6 show decoupled spectra. The undecoupled spectrum in Figure 7 shows the multiplicities of the signals. The assignments of the signals are listed in Table V.

Table V

 ^{13}C -NMR

Signal	Chem. shift ppm vs. TMS	Multiplicity	Number of carbons	Species
1	55.9	t,m	1	$\diagup\text{N}-\text{CH}_2-\text{N}\diagdown$
5	118.9	d,d,m	1	arom. $\text{C}-\text{H}$
9	120.8	t (bro)	1	arom. $\text{C}-\text{SO}_2-\text{NH}-\text{CH}_2$
8	127.3	d (bro)	1	arom. $\text{C}-\text{H}$
6	129.6	d,d	1	arom. $\text{C}-\text{Cl}$
7	135.9	d,d	1	arom. $\text{C}-\text{SO}_2\text{NH}_2$
10	147.7	d,m (bro)	1	arom. $\text{C}-\text{NH}-\text{R}$

d: doublet; t: triplet; m: multiplet;
(bro): broadened

NMR spectrum no. 74533

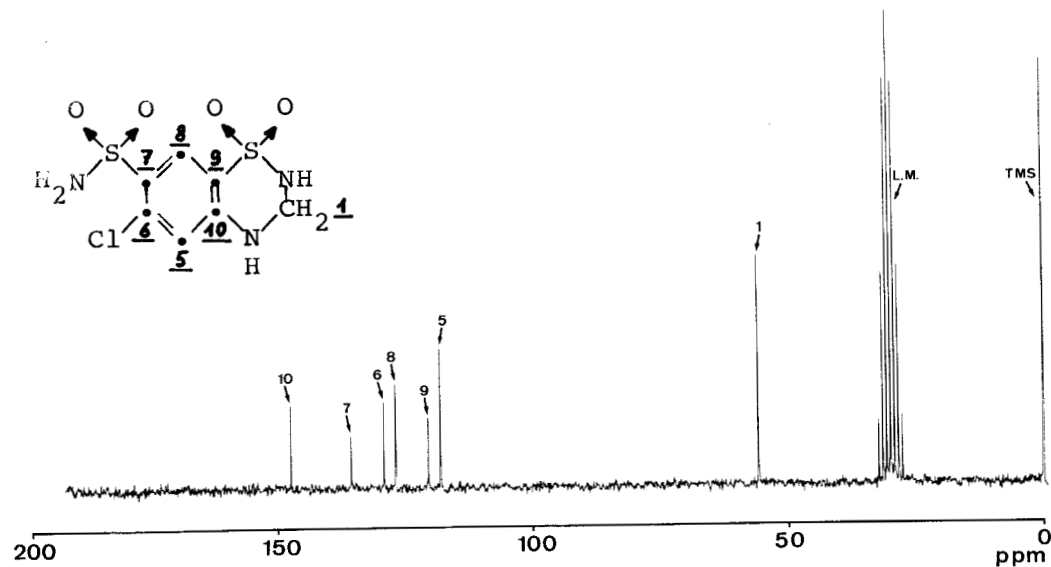


Figure 5. ^{13}C -NMR spectrum of hydrochlorothiazide

NMR spectrum no. 74538 A

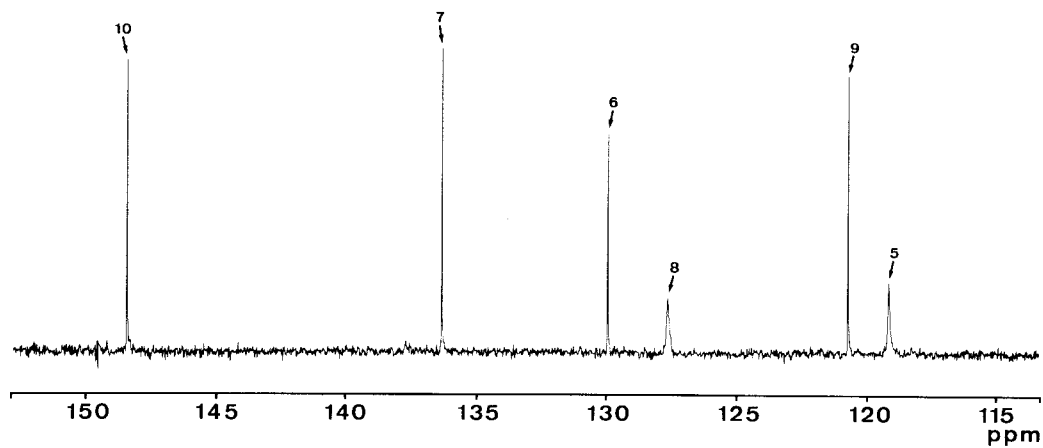
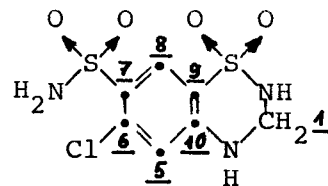


Figure 6. ¹³C-NMR spectrum of hydrochlorothiazide

NMR spectrum no. 74538 B

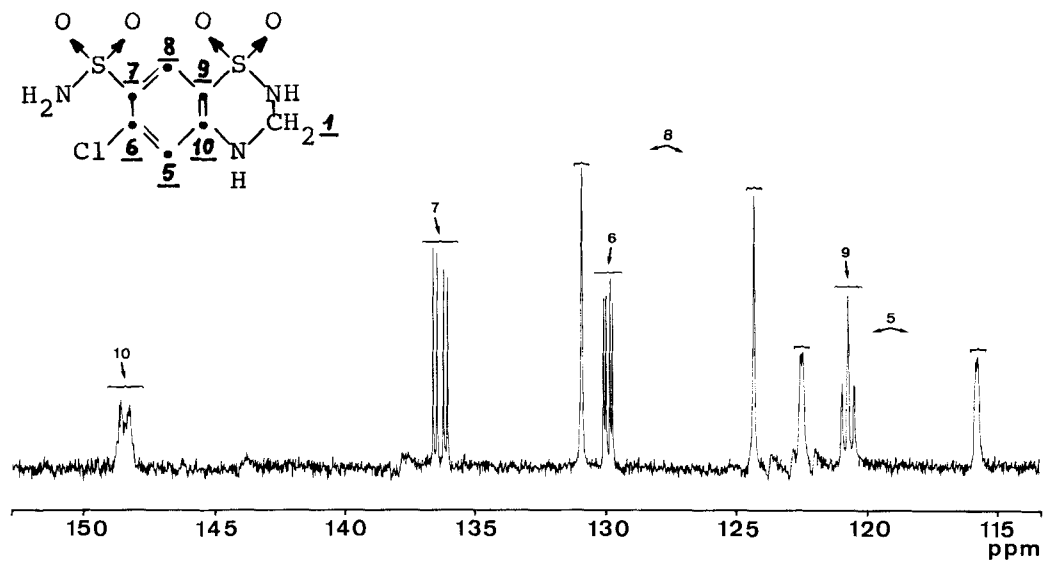


Figure 7. Undecoupled ^{13}C -NMR spectrum of hydrochlorothiazide

2.1.6 Mass(11)

Spectra were recorded on a Varian CH7 mass spectrometer using the direct inlet system, 70eV electron energy and an ion source temperature of 180°C. At a sample temperature of about 240°C the molecular ion m/e 297 could be detected but the spectrum was complicated and difficult to interpret due to pyrolytic degradation. Routine mass spectroscopy is considered to be inadequate for the characterization of hydrochlorothiazide.

2.2 Physical Properties of the Solid State

2.2.1 Thermal Analysis(15)

Melting point

Melting points reported in literature (5,14,16,17,18,19,20) vary within the temperature range of 263 to 275°C. The strong dependence of the melting point on heating conditions has been confirmed with the Mettler FP-2 hot stage microscope as well as with the Perkin-Elmer DSC-2. The effect which causes the anomalous melting behaviour(20) is not clearly understood.

Differential Scanning Calorimetry

The melting point of hydrochlorothiazide according to DSC-2 measurements, varies from 266.0°C for a scan speed of 1.25°C min⁻¹ to 273.3°C for a scan speed of 80°C min⁻¹ (Figure 8). As any effect (e.g. decomposition or transition into another crystal modification) is suppressed at a high scan speed, one can regard the value for an infinitely fast scan speed as the true melting point. Extrapolation of the curve shown in Figure 8 gives a melting point of 274.5 ± 0.3°C*. This agrees well with the melting point reported in The Merck Index(5). However, the purity values obtained from melting curves measured with the DSC-2 are independent of the scan speed as well as of the surrounding atmosphere. When a sample of hydro-

* Error of the mean value in terms of confidence intervals on a 95 % level

chlorothiazide was tested at scan speeds of 1.25, 2.5, 5 and 20°C min⁻¹ the mean value obtained for the purity was 99.3 ± 1.0 mole per cent. A typical DSC melting curve is shown in Figure 9. A slight exotherm, indicating decomposition in the liquid phase, is seen above the melting point.

Thermogravimetry

Measurements were performed with a Perkin-Elmer thermobalance TGS-1. The results were:

- less than 0.1 % volatile impurities up to a temperature of 280°C.
- decomposition starts at 307°C.

2.2.2 Density

1.68 ± 0.01 g cm⁻³ (19,21)

2.2.3 X-ray Diffraction

Studies by Dupont and Dideberg(19) on a single crystal measuring 0.2x0.4x0.1 mm gave the following crystallographic data

System:	Monoclinic
Space group:	P2 ₁
Unit cell:	Z = 2 molecules
	a = 7.419 ± 0.006 Å
	b = 8.521 ± 0.003 Å
	c = 10.003 ± 0.002 Å
	β = 111.720°
	V = 587.5 Å ³

Calculated density: 1.672 g cm⁻³

Linear absorption coefficient μ = 6.71 cm⁻¹ (Mo K_α: 0.7107). These results, found on a crystal crystallized from ethanol, correspond well with those found on a sample crystallized from methanol in a preliminary study(22).

A powder diagram, calculated from the single crystal data, agrees very well with measurements on industrial production lots. The powder diffraction pattern, as shown in Table VI, was obtained with a Guinier-DeWolf No. 2 camera with CuK_α (1.54178 Å) radiation(23).

Figure 8. Melting point of hydrochlorothiazide as a function of scan speed (DSC)

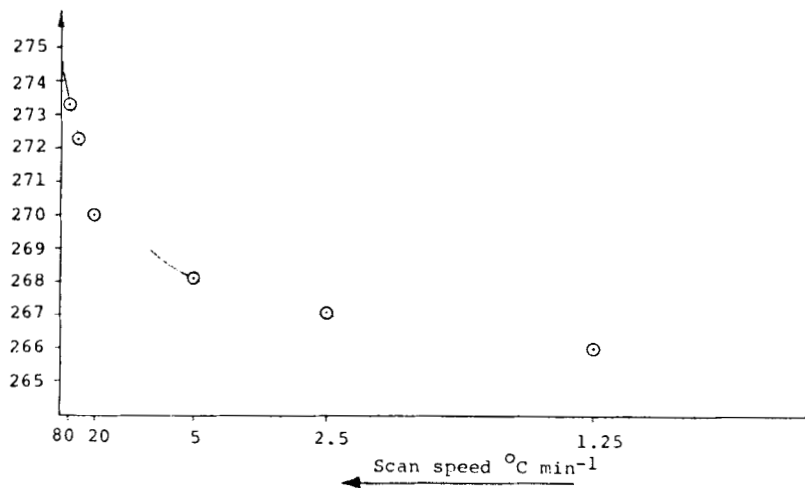


Figure 9. DSC melting point curve of hydrochlorothiazide

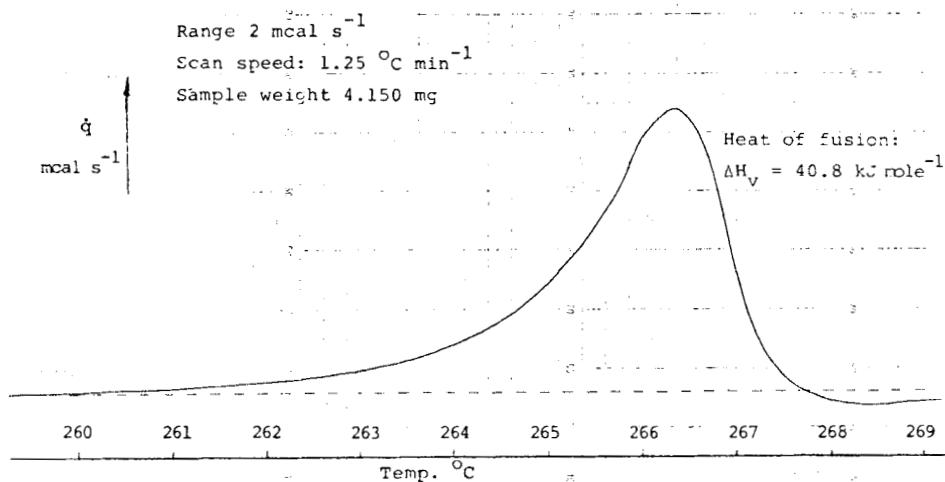


Table VI

X-ray diffraction pattern of hydrochlorothiazide powder

d(Å)	Intensity	d(Å)	Intensity
9.3	very weak	2.71	very weak
6.9	weak	2.67	strong
6.3	very weak	2.62	medium
5.35	very strong	2.50	weak
4.75	strong	2.45	moderate
4.65	strong	2.40	very weak
4.26	very strong	2.38	very weak
4.15	very strong	2.35	very weak
4.09	very weak	2.29	moderate
3.87	moderate	2.25	very weak
3.62	very strong	2.21	very weak
3.44	moderate	2.19	very weak
3.39	weak	2.16	weak
3.19	strong	2.12	weak
3.14	weak	2.07	very weak
3.10	strong	2.06	very weak
2.89	weak	2.03	moderate
2.74	weak		

2.3 Solubility

2.3.1 Solubility in Homogeneous Media

Hydrochlorothiazide is soluble in aqueous solutions of inorganic bases like sodium hydroxide(6) or ammonium hydroxide(5) and in organic bases like n-butylamine(6). Solubilities in aqueous solutions are given in Table VII, and in some commonly used organic solvents, in Table VIII.

The surface tension of the saturated aqueous solution at 23°C was found to be 724 μN per cm by Lerk and Lagas(21). The increase of the solubility upon addition of non-ionic surfactants was studied by Aboutaleb et al.(24).

Table VII

Solubility in aqueous solutions(13)

Solvent	t°C	pH of the solution	Solubility g in 100 ml solution
water	25	6.2	60.9 . 10 ⁻³
water	37	7.2	108 . 10 ⁻³
0.9 % NaCl	25	6.1	59.4 . 10 ⁻³
0.1 N HCl	25	1.0	60.8 . 10 ⁻³
0.1 N acetic acid	25	2.9	63.6 . 10 ⁻³
0.1 N acetic buffer pH 4.4	25	4.5	62.3 . 10 ⁻³
0.067 M phosphate buffer pH 7.4	25	7.4	61.6 . 10 ⁻³
0.05 M borate buffer pH 9.0	25	8.9	103 . 10 ⁻³
1 M ammonia(25)	25	11.6	2.2
0.1 N NaOH	25	10.2	1.79
simulated gastric fluid pH 1.1	37	1.1	108 . 10 ⁻³
simulated intestinal fluid pH 7.4	37	7.5	109 . 10 ⁻³

Table VIII

Solubility in non aqueous solvents

Solvent	temp.°C ca.	Solubility g in 100 ml solution
acetone	25	13.7 (25)
acetic acid	25	0.15 (25)
acetonitrile	25	2.0 (25)
ethylacetate	25	0.59 (25)
chloroform	23	0.1 (13)
ethanol (96 %)	23	1.3-1.4 (13)
methanol	23	3.9-4.1 (13)
dichloromethane	23	< 0.02 (13)

2.3.2 Partition Coefficients

The partition between n-octanol and aqueous phases at 25°C is exemplified by the following data(13)

0.1 N	HCl (pH 1.06):	pC_{org}/C_{aq}	=	1.94
0.1 M	glycine			
	buffer (pH 3.0):	pC_{org}/C_{aq}	=	0.866
0.067 M	phosphate			
	buffer (pH 7.4):	pC_{org}/C_{aq}	=	0.855

2.4 Ionisation in Aqueous Solution

The ionisation constants quoted in the literature differ. The values reported by Mollica et al.(26) and by Stahl agree best with the solubility behaviour(13). They are listed below in Table IX.

Table IX

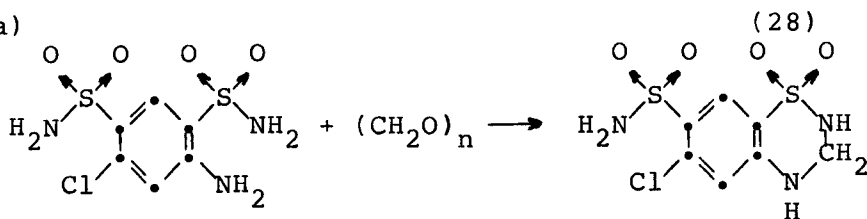
pK-Values in aqueous solutions

pK-Value		Method
pKa 1	8.81 \pm 0.05	photom. titr. (13)
pKa 2	10.4 \pm 0.1	photom. titr. (13)
pKa 1	8.6	potent. titr. (26)
pKa 2	9.9	potent. titr. (26)
pKa	8.7	spectrophotom. (26)

3. Synthesis

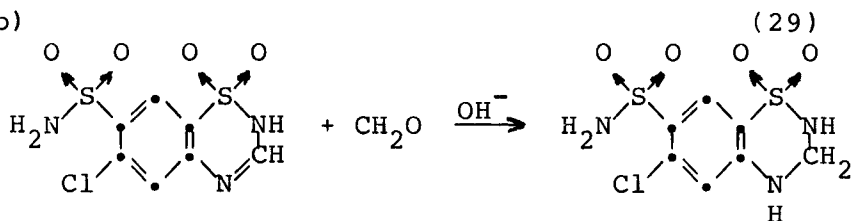
According to Kleemann(27) two ways of synthesis are used

a)



5-Chloro-2,4-disulfamoylaniline and paraformaldehyde react in non aqueous media to give hydrochlorothiazide.

b)



6-chloro-7-sulfamoyl-2H-1,2,4-benzothiadiazine-1,1-dioxide reacts with formaldehyde in aqueous alkaline solution to form hydrochlorothiazide.

4. Stability and Degradation

4.1 Bulk Stability(25)

Hydrochlorothiazide stored at room temperature for five years shows no degradation and heat affects it very slowly, e.g. treatment for 2 hours at 230°C gives a yellowish discoloration but no significant change of the physical properties. Although hydrochlorothiazide is fairly stable in normal daylight, it should not be exposed to intense light: 48 hours at 180 000 Lux (Xenotest) destroyed about 3 per cent of a sample spotted on glass fibre paper.

4.2 Solid-Solid Interactions

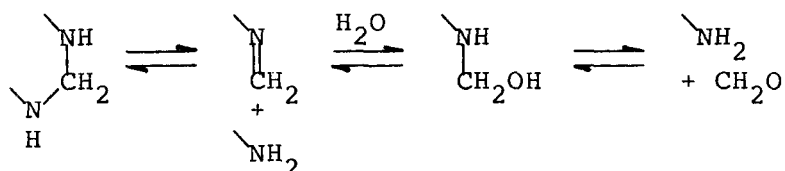
Bornstein and Lach(30) found that hydrochlorothiazide reacts under the influence of humidity with adjuvants containing metal compounds. The changes in UV-absorption spectra obtained by diffuse reflectance spectrometry were interpreted as the result of charge-transfer chelation. In a compatibility study with Aerosil 2000, calcium stearate and talc using diffuse reflectance spectroscopy, tlc and UV spectroscopy after extraction, no indications of degradation under usual manufacturing and storage conditions were found(13).

4.3 Stability in Solution

In aqueous solutions, hydrochlorothiazide undergoes hydrolysis to give formaldehyde and

6-Chloro-2,4-disulfamoylaniline. The dependence of the reaction rate on temperature and pH was studied by Mollica et al.(26,31) and Yamana et al.(32). Between pH 2.5 and pH 11.5 the rate follows a bell shaped curve with a maximum at about pH 7.2. Below pH 2 and above pH 12 the reaction rate increases rapidly.

This pH rate profile and the lack of significant buffer catalysis was explained by Mollica by the formation of intermediates of the imine type and, upon hydration, of the hydroxymethylamine type(26).



5. Methods of Analysis

5.1 Elemental Analysis(33)

Element	C	H	Cl	N	O	S
% calculated	28.24	2.71	11.91	14.12	21.49	21.54
% found	28.21	2.72	12.15	14.18	21.26	21.45

5.2 Identification

Chemical tests were described by Kertesz(34) Kala(35) and Perez(18). Microchemical identification methods were reported by de Zoeten(36), Groenewegen(37), Kala(38) and Auerbach(39). Usually, hydrochlorothiazide is identified by spectroscopic means, e.g. by its IR (6,14,40) and UV (6,14,40,41) spectra or by one of the chromatographic techniques cited in section 5.9.

5.3 Colorimetry

Hydrochlorothiazide is rapidly hydrolysed in acid or alkaline solutions. By diazotisation of the hydrolysis product 5-chloro-2,4-disulfamoylaniline and subsequent coupling with an aromatic amine or a phenol, stable azo dyes are produced.

N-(1-naphthyl)-ethylenediamine(42,43,44), chromotropic acid(45), guajacol sulfonic acid(46), and thymol(35) have been reported as coupling agents. These methods are considered to be suitable for the analysis of pharmaceuticals and may also serve as low cost techniques in biopharmaceutical studies. Other methods are based on condensation reactions of the hydrolysis products(26,47) or on direct color reactions of hydrochlorothiazide with different reagents in the presence of concentrated sulfuric acid(18,42,48).

Recently, Elsayed and Nwakanma(49) reported on ion pair extraction with safranin basic dye as a simple, selective and sensitive new method for tablet analysis.

5.4 Ultraviolet Spectrophotometry

Rehm and Smith(45) showed that UV-spectrophotometry is not suitable for the determination of hydrochlorothiazide in the presence of its hydrolysis product 5-chloro-2,4-disulfamoyl-aniline. In spite of this limitation, the method was described for tablet analysis by Steinbach, Moeller(50) and Ruiz Rodriguez et al.(51). Fazzari combined UV-Spectrophotometry with a column extraction technique(52). The specificity of the method may also be improved by calculating the concentrations from pH-induced spectral changes(53,54,55).

An automated determination of hydrochlorothiazide in single multicomponent tablets was described by Urbanyi and O'Connell(56). The determination of hydrochlorothiazide in (bovine) serum is possible after separation by dialysis(57).

Qualitative results were obtained by UV-spectrometry of polyethylene container material(58) and by means of diffuse reflectance spectroscopy on powder mixtures(30). UV-spectrophotometry is also applied in dissolution rate studies, e.g. in USP XIX(6).

5.5 Phosphorimetry

Bower and Winefordner(59) reported on a technique for room temperature phosphorescence measurements on hydrochlorothiazide and found it to be a simple and selective method suited to certain clinical analyses.

5.6 Fluorimetry

Schäfer, Geissler and Mutschler(60) developed two methods based on fluorimetric measurement on tlc plates. By coupling the diazotised hydrolysis product of hydrochlorothiazide to a fluorescent compound, followed by chromatographic separation, 0.6 ng of 6-chloro-2,4-disulfamoyl-aniline could be detected. Because the authors found the hydrolysis step difficult to reproduce quantitatively they recommend measurement of the fluorescence of underivatised hydrochlorothiazide. The sensitivity of the latter method is lower but still sufficient for the analysis of human plasma, urine and saliva after oral administration of 25 mg hydrochlorothiazide.

5.7 Polarography

Cohen et al.(61) and Woodson and Smith(62) reported on the polarographic response of hydrochlorothiazide and related compounds. Practical applications were described in USP XVIII(63) for tablets and in a paper of Kkolos and Walker(64) for multicomponent tablets. The polarographic determination is reported to be suitable for single tablet analysis without separation of other components.

5.8 Titration

The titration of the pure compound with strong bases in non aqueous solvents has found widespread application(35,65,66,67,68). USP XIX(6) titrates hydrochlorothiazide with sodium methoxide in n-butylamine with azo violet as indicator. BP 73(8) describes a potentiometric titration with tetra-butylammoniumhydroxide in pyridine.

Other titration techniques applied to hydrochlorothiazide active substance or formulations are listed below:

Complexometric after precipitation with Pb^{++} (69) or with Hg^{++} (70,71). Amperometric with nitrite(72). Bromatometric(73). Thermometric with sodium hydroxide(74). Sulfate determination after mineralisation(75). Argentometric after mineralisation(76).

5.9 Chromatography

5.9.1 Thin Layer Chromatography

Paper chromatography was of some importance for the identification of hydrochlorothiazide (17,40,77,78,79,80) before it was replaced by thin layer chromatography. In tlc, silica gel layers are the most often used sorbents. They were shown to be suitable for qualitative(79-91) and quantitative(50,60) analysis of hydrochlorothiazide in pharmaceuticals(50,79,87) and in biological material(60,88,90,91). Other sorbents like aluminum oxide(80,83,87) and cellulose(81) therefore have not received much attention.

The detection of hydrochlorothiazide on paper (17,40,78), cellulose layers(81) and aluminum oxide(80,83,87) was not studied intensely. On silica gel, the quenching of fluorescence on layers containing a fluorescence indicator (detection limit 0.2 μg) and colour reaction by hydrolysis, diazotisation and coupling with sodium chromotrope (detection limit below 0.1 μg) were found to be reliable and sensitive methods. Other reported visualisation techniques(81,85,87,88,92) were not found to be of comparable sensitivity.

Successfully tested systems (examples) (93):

- Ethyl acetate+chloroform+methanol (11+8+1), at about 23°C, on silica gel 60 F-254 (Merck), R_f of hydrochlorothiazide about 0.3. Used for semi-quantitative stability tests on dosage forms.
- Two step development system.
 - a) Diethylether+chloroform+ethyl acetate+methanol (10+8+6+1.5),
 - b) Ethyl acetate+chloroform (22+3), at about 23°C, on silica gel Sil-G 25 HR UV 254 (Macherey-Nagel), $R_{f_a} + R_{f_b}$ of hydrochlorothiazide about 0.6. Used for purity testing on active substance.

The direct quantitation of hydrochlorothiazide on tlc plates was reported by Steinbach et al.(50) (densitometry at 272 nm) and Schäfer et al.(60) (fluorimetry). The latter method allows the determination of 2 ng hydrochlorothiazide on the plate and was used for the analysis of human body fluids after single dose application.

5.9.2 Liquid Chromatography

Separation of hydrochlorothiazide from tablet ingredients by chromatography on alkaline celite columns(52,94) and from basic compounds in multi-component pharmaceuticals on ion exchange columns (56,95) was shown to be possible under low pressure conditions (gravity).

Later on, high pressure liquid chromatography replaced the low pressure methods completely. The diversity of reported HPLC methods is illustrated by table X.

5.9.3 Gas Chromatography

The determination in plasma, blood corpuscles and urine by gas chromatography was reported by Lindstroem et al.(104,105). Hydrochlorothiazide was methylated with methyl iodide, using the extractive methylation procedure. For the quantitative evaluation, an internal standard, chlor-thalidone, was used.

Chromatographic conditions(104):

Column: 1 % SE-30 on Gas-Chrom Q (80-100 mesh),
225°C: Carrier nitrogen
Injector: 230°C
Detectors: ECD, 300°C; FID, 270°C

The method has been applied e.g. by Beermann et al.(106-113) in pharmacokinetic and bioavailability studies and by Wallace et al.(88) as a confirmatory method to tlc identification methods. Vandenheuvel et al.(114) developed a method for the analysis of blood and plasma based on the 'on-column methylation technique' with tetramethyl-anilinium hydroxide and the use of 6-bromo-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide as the internal standard. The same internal standard was used by Redalieu et al.(115) in a modification of the Lindstroem method.

5.10 Electrophoresis

Ruggieri(116) proposed electrophoretic separation of hydrochlorothiazide for quantitative analysis.

Table X HPLC methods

column	eluent:	volumes	sample	ref.
CSP anion exch. on Zipax 30 μ m 1000x2.1 mm	0.005 Na ₂ SO ₄ in pH 9.2 borate buffer:	35	H.+hydrolysis product	96
	methanol:	5	+hydralazine	97
Corasil-C ₁₈ 220x2.3 mm	screening		artificial mixtures of antihypert.	98
Corasil-phenyl 1220x2.3 mm				
μ Bondapak C ₁₈ 300x4 mm	0.01 M aq. NaH ₂ PO ₄ :	4	serum/urine	99
	methanol:	1		
Spherisorb ODS 10 μ m, 250x3 mm	water:	85	serum (gel filtered)	100
	methanol:	15		
Lichrosorb SI60 5 μ m, 250x2.1 mm	n-hexane:	77	tablets (H.+reserpine)	101
	2-propanol:	18		
	chloroform:	5		
	diethylamine:	0.01		
Lichrosorb 5 μ m, 500x4.4 mm	n-hexane:	55	serum	102
	ethanol:	45		
Nucleosil 10-CN 10 μ m, 200x4.8 mm	0.01 M aq. C ₁₂ H ₂₅ NaO ₄ S:	75	tablets H.+reserpine +hydralazine	103
	2-propanol:	20		
	0.1 N aq. H ₂ SO ₄ :	5		

H. = hydrochlorothiazide

6. Interferences of Hydrochlorothiazide in Analytical Methods

Hydrochlorothiazide interferes with the urinary estriol determinations by gas chromatography(117) and by colorimetry with the Kober reaction (118).

7. Pharmacokinetic and Metabolic Studies

7.1 Analytical Methods Used for Biological Material

Besides radiometric procedures used in absorption and distribution studies(77,106,119, 120), several colorimetric methods were developed for the determination of hydrochlorothiazide in plasma and urine(42-44,121-124). The performance of bioavailability studies by colorimetric methods may produce errors as could be seen for chlorothiazide(125). Therefore gas chromatographic methods were predominantly used, i.e. the method of Lindstroem et al.(104) in kinetic studies of the group of Beermann (107-113) and by Jordoe et al.(126), the method of Vandenheuvel et al.(114) used by Sundquist et al.(127) and the procedure of Redalieu et al.(115). High-pressure liquid chromatography(100) and fluorimetric(60) or spectrophotometric(128) determination after separation by tlc may be also suitable for the determination of hydrochlorothiazide in biological material.

7.2 Absorption

After single intravenous or oral administration of ^{14}C -labelled hydrochlorothiazide (Doses: i.v. 1, 35, 65 mg, n = 3; p.o. 5, 50, 65 mg; n = 4, n = 6) to volunteers and patients 90-93 % and 53-83 % of dose, respectively were excreted in urine. Therefore absorption of an oral drug dose was in the range of 60-80 %. It was found to be reduced in patients with congestive heart failure or renal and hepatic diseases since only about 40 % of dose or less were eliminated renally(106).

Hydrochlorothiazide was excreted in urine of rat or man almost completely as the intact substance(77,106).

The peak plasma concentrations of total radioactivity in patients ($n = 3$) were 260, 386 and 616 ng/ml reached within 3-4 hours, the corresponding values in blood cells were about 3 times higher(106). Similar blood/plasma concentration ratios were also reported for volunteers(106,114, 115). The nature of the binding of hydrochlorothiazide in the erythrocytes is still unknown. In vitro experiments with bovine carboanhydrase showed no binding(104,106).

7.3 Distribution

Organ distribution pattern in rats after single oral administration of tritium labelled hydrochlorothiazide (dose: 5 mg) revealed highest concentrations of total radioactivity in liver (27.8 $\mu\text{g/g}$) and gastro-intestinal tract (36.0 $\mu\text{g/g}$) within 1 hour after dosing. At the same time the concentration in plasma was 1.53 $\mu\text{g/ml}$, that in spleen, muscle and brain 0.36-0.46 $\mu\text{g/g}$ (77).

A low degree of hydrochlorothiazide binding to bovine serum albumin was obtained with only one binding site class(57).

7.4 Basic Pharmacokinetics

In volunteers after single oral dose administration of hydrochlorothiazide ($n = 8$; doses: 12.5, 25, 50, 75 mg) the peak plasma concentrations of intact drug reached within 1.5-5 hours and the area under the concentration curves (AUC, 0-9 hours) were linearly correlated with the dose. Peak concentrations in dependence of the increasing doses were 70 ± 19 , 142 ± 50 , 260 ± 88 and 376 ± 70 ng/ml, respectively ($\bar{x} + s_x$). Hydrochlorothiazide was eliminated from plasma mostly in a biphasic way with terminal half-lives of 5.6-14.8 hours(107). In the same volunteers using the same experimental design the urinary excretion and the dose administered were significantly correlated too. At oral doses of 12.5, 25, 50 and 75 mg the urinary excretion (0-48 hours) was 8.5 ± 2.0 , 17.9 ± 4.2 , 33.4 ± 8.6 and 48.9 ± 7.6 mg, respectively. The cumulative urinary recovery of the drug was 65-72 % of dose for all doses administered. Renal clearance was also independent of dose with

345 + 123 to 319 + 86 ml/min(107). In seven patients with congestive heart failure (dose: 50 mg, n = 6; 75 mg, n = 1) highest concentrations of intact hydrochlorothiazide in plasma were found within 1.5-8 hours with 282-672 ng/ml. The terminal half-life in plasma was 8.9-28.9 hours (n = 6) and 3.1 hours in one patient with the highest heart failure. Urinary excretion of intact drug (0-7 days) in these patients may be reduced (20.8-71.6 % of dose)(111).

In hypertensive patients during repeated treatment with different doses of hydrochlorothiazide (doses: 12.5, 25, 50, 75 mg/day for 2 consecutive weeks; 75 mg for additional 4 weeks) pre-dose plasma levels of intact substance showed a linear relationship to increasing doses with 15 + 7, 17 + 8, 27 + 11 and 34 + 17 ng/ml, respectively. This was also obtained for plasma concentrations 5 hours after dosing. Steady state plasma concentration of intact drug after 6 weeks of daily treatment with 75 mg hydrochlorothiazide was found to be 111 ng/ml. Urinary excretion of intact hydrochlorothiazide within the last 24 hours of each treatment period was about 60 % of dose and renal clearance accounted for 317 + 120 ml/min(113).

Other authors, using a less specific method, have observed steady state plasma levels of 970 + 90 ng/ml and 2250 + 20 ng/ml ($\bar{x} \pm s_{\bar{x}}$) during a 12 week and 20 week treatment period in hypertensive patients at daily oral doses of 150 and 450 mg hydrochlorothiazide, respectively(44).

7.5 Bioavailability

Since hydrochlorothiazide is excreted almost completely as the intact substance in man, its cumulative urinary excretion is the best measure of bioavailability.

As could be seen above, the urinary recovery of the drug after single oral doses of 12.5-75 mg hydrochlorothiazide (comm. 25 mg tablets) is independent of the dose with 65-72 %(107).

The urinary excretion was in the same range in an experiment where bioequivalence of two dosage forms (comm. 25 mg tablets of different origin) was observed with 70.8 + 14.9 vs. 65.2 + 10.1 % of dose (108). This was also observed in studies comparing

several dosage forms of hydrochlorothiazide, but less specific colorimetric methods were used(121, 123,124).

Enhanced bioavailability of hydrochlorothiazide was obtained in volunteers ($n = 8$; dose 75 mg) when the drug was administered with food (74.2 ± 6.5 vs. 63.2 ± 8.0 % of dose measured in urine) after the pretreatment of volunteers with the anticholinergicum propantheline ($n = 6$; dose 75 mg; 66.9 ± 4.4 vs. 49.3 ± 5.3 % of dose in urine)(109,110) or after concomitant administration of polyvinylpyrrolidone(128).

No influence on the bioavailability of hydrochlorothiazide was observed when sotalol, metoprolol or hydralazine were administered in combined dosage forms or separately to volunteers(122, 126,127,129).

A significant malabsorption, measured in terms of urinary excretion of intact hydrochlorothiazide was found in patients with congestive heart failure ($n = 7$; dose: 50-75 mg) or in patients after intestinal shunt surgery ($n = 5$; dose 75 mg) with an average urinary recovery of 40.7 and 30.7 % of dose, respectively(111,112).

The relationship between bioavailability data and in vitro dissolution test results has been investigated repeatedly(121,123).

8. Acknowledgements

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KETOPROFEN

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1. Description

1.1 Nomenclature

1.1.1. Chemical Names

m-Benzoylhydratropic acid (1,2),
 α -(benzoylphenyl) propionic acid (3),
 α -(3-benzoylphenyl) propionic acid (4,5,6),
 2-(3-benzoylphenyl) propionic acid (1,2,7-11),
 2-(benzoyl-3-phenyl) propionic acid (12,13),
 Benzeneaceticacid, 3-benzoyl- α -methyl. The latter
 name is used by Chemical Abstracts. The Chemical
 Abstract's registry number for (\pm)ketoprofen is
 22071-15-4 , for the (+) enantiomer it is
 22161-81-5 and for the (-) enantiomer it is
 56105-81-5 .

1.1.2. Nonproprietary Name

Ketoprofen

1.1.3. Propietary Name

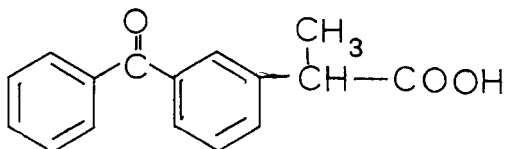
Orudis[®], Alrheumat[®], Alrheumun[®],
 Profenid[®].

1.2 Formula

1.2.1. Empirical

$C_{16}H_{14}O_3$

1.2.2. Structural



1.3 Molecular Weight

254.29

1.4 Appearance, Colour, Odour and Taste

A slightly coloured, odourless, tasteless powder with an irritant dust.

2. Physical Properties

2.1 Melting Range

93-95°C(5), 94-95°C(14), 94°C(1,2,15,16) , 96°C, 92°C(17), 91°C(18).

2.2 Solubility

ether	soluble	(13,14)
ethanol	soluble	(4,14)
water	slightly soluble	(5)
octanol	soluble	(5)
disopropyl ether	soluble	(5)
acetone	soluble	(14)
chloroform	soluble	(14)
dimethylformamide	soluble	(14)
methanol	soluble	(14)
ethyl acetate	soluble	(13)

2.3 pH

The pH of a 3.95×10^{-4} M solution in water is 6.5 (5).

2.4 Dissociation Constant

The pKa in : dioxan : water (2:1) is 7.2 (20), acetonitrile : water (3:1) is 5.02 (5), methanol : water (3:1) is 5.937 (14).

2.5 Partition Coefficient

The partition coefficient of ketoprofen in an n-octanol/water (phosphate buffer pH 7.35 and initial ketoprofen concentration of 0.2542 mg/ml in this) is 0.105 (5) and at pH 7.4 (MacIlvaine's buffer and initial ketoprofen concentration of 0.0240 mg/ml in this) is 0.97 (20). At these pH's most of the ketoprofen is ionised (20) and thus an increase in the initial concentration of ketoprofen in the buffer will cause an alteration in the partition coefficient.

2.6 Thermal Analysis

2.6.1. Differential Thermal Analysis

A D.T.A. thermogram of ketoprofen at a heating rate of 5°C per minute and sample size of 4 mg in a static air atmosphere shows an endotherm at 96°C which indicates melting (Fig. 1). If the melted sample is cooled to 0°C and then analysed again no peak corresponding to melting can be detected. The ketoprofen is in a glass like form. On storage the glass like form changes to the regular crystalline form, conversion is complete in ten days at room temperature.

2.6.2. Thermogravimetric Analysis

A TGA thermogram of ketoprofen at a heating rate of 2°C per minute and sample size of 6 mg in a static air atmosphere shows no loss of weight until 223°C when ketoprofen decomposes (Fig.2).

2.7 Crystallinity

2.7.1. Polymorphism

Ketoprofen can exist in two Polymorphs as mentioned in section 2.6.1. on differential thermal analysis. Ketoprofen forms white crystal prisms when crystallised from di-isopropyl ether (5).

2.8 Ultraviolet Spectrum

The UV spectra of ketoprofen (3.95×10^{-4} M) in the following solvents are given in Figure 3 (using Varian Techtron M 165)

1. 0.1N hydrochloric acid pH 1.2 (5)
2. distilled water pH 6.5 (5)
3. 0.1N sodium hydroxide pH 12.9 (5)

The λ_{\max} appears at 261 nm and corresponds to a K band. This maximum is independent of pH but the maximum absorbance is slightly decreased with increasing pH. The λ_{\max} in methanol has been reported as 255 nm and $\log \epsilon = 4.33$ (14,21). The λ_{\max} in ethanol has been reported as 255 nm and

$E_{1\%}^{1\text{cm}}$ 640 (7).

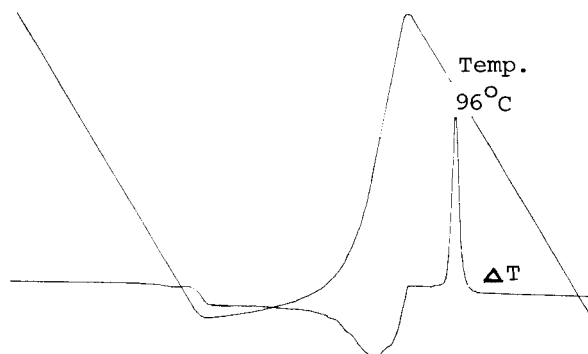


Figure 1. Differential Thermogram of Ketoprofen

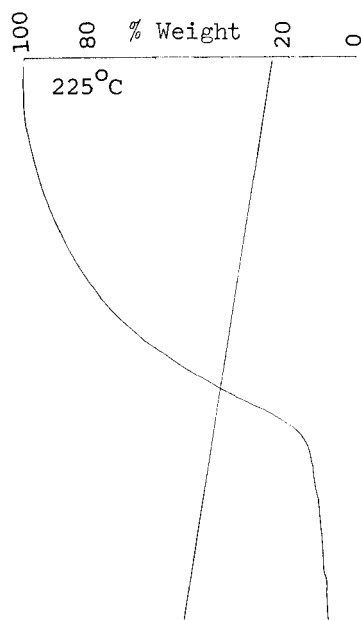


Figure 2. Thermogravimetric Curve of Ketoprofen

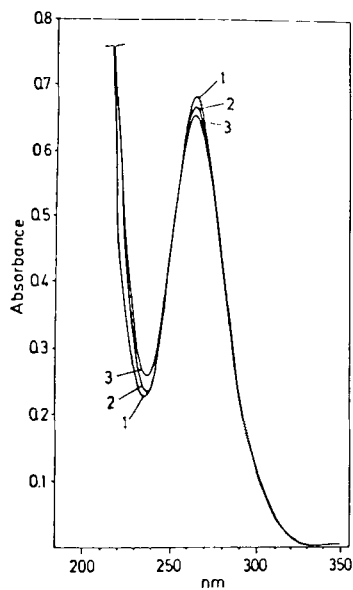


Figure 3 Ultraviolet Spectrum of Ketoprofen
(see text for key)

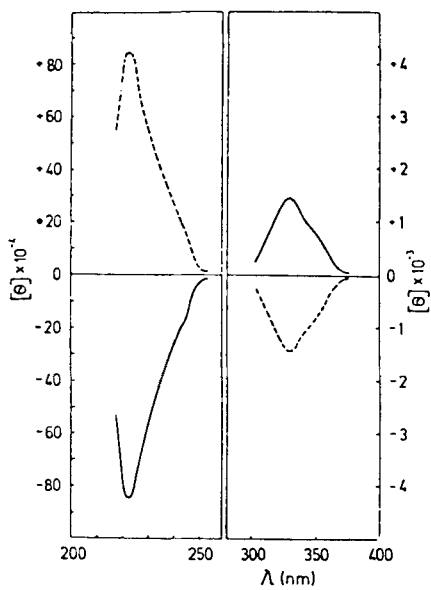


Figure 4 CD Spectra of Ketoprofen's Enantiomers

2.9 Optical Rotation

Ketoprofen is a racemic mixture of (\pm)- α -(3-benzoylphenyl)-propionic acid (3,4,5). Both enantiomers show Cotton Effects at 223 nm (4) as demonstrated in Figure 4. The (+)-enantiomer shows a positive Cotton Effect indicating a S-absolute configuration and interacts more strongly with human serum albumin as well as with biotransformation enzymes than the (-)-enantiomer (4).

(+)-enantiomer $[\alpha]_D^{23} + 57.1^\circ$ (C = 0.76 in CH_2Cl_2) (4,5)

(-)-enantiomer $[\alpha]_D^{23} - 57.4^\circ$ (C = 0.88 in CH_2Cl_2) (4,5)

(+)-enantiomer $[\alpha]_D^{23} + 49.6^\circ$ (C = 1.15 in CH_2Cl_2) (3)

(-)-enantiomer $[\alpha]_D^{23} - 52.4^\circ$ (C = 1.05 in CH_3Cl_3) (3)

2.10 Mass Spectrum

The mass spectrum of ketoprofen has not been published but the mass spectrum of the methyl ester has (8,10,13), see Figures 5 and 6 (using LKB900S gas chromatograph mass spectrometer) (13). The fragmentation pattern is reported in Figure 7. The fragmentation pattern for ketoprofen will be similar, the methyl of the ester being replaced by a hydrogen atom.

2.11 Photoelectronic Spectrum

The photoelectronic spectrum on a Vacuum Generators UV G3 instrument (Figure 8) exhibits several bands characteristic of the benzophenone group. Notably the partially overlapping bands at 9.07 and 9.45 eV, these bands are a result of the ionisation of the two degenerated phenyl orbitals. The energy at 10.62 eV corresponds to the ionisation of a free electron pair of the carboxylic carbonyl group.

Bands have been assigned as:-

$4\pi \left(n(a'') \right) < n_O < 2\pi_{CO} < n_O(a'')_{\text{keto}} < \sigma_{\text{onset}}$ (5)

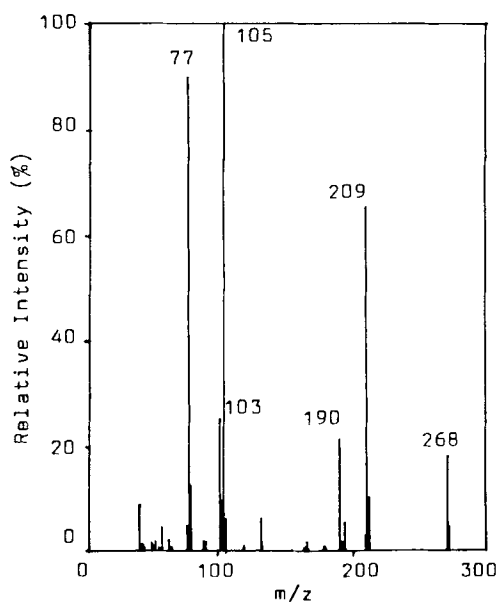


Figure 5 Mass Spectrum of Ketoprofen Methyl Ester at 70 EV

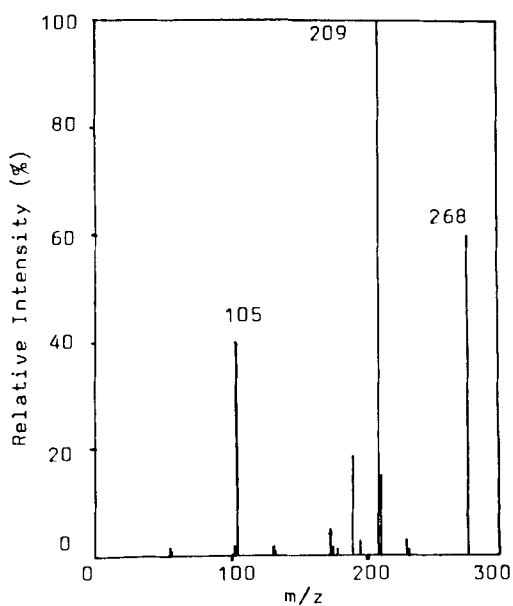


Figure 6 Mass Spectrum of Ketoprofen Methyl Ester at 20 EV

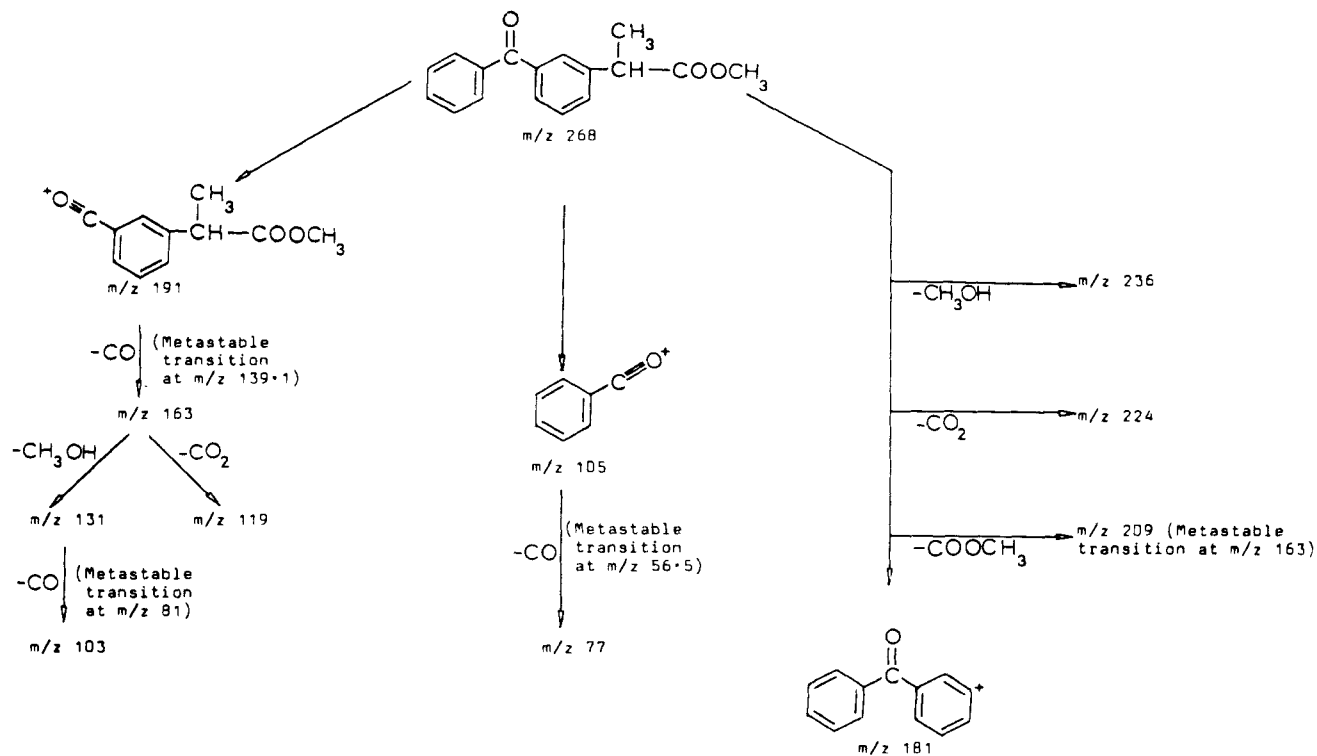


Figure 7 Fragmentation Pattern of Ketoprofen Methyl Ester

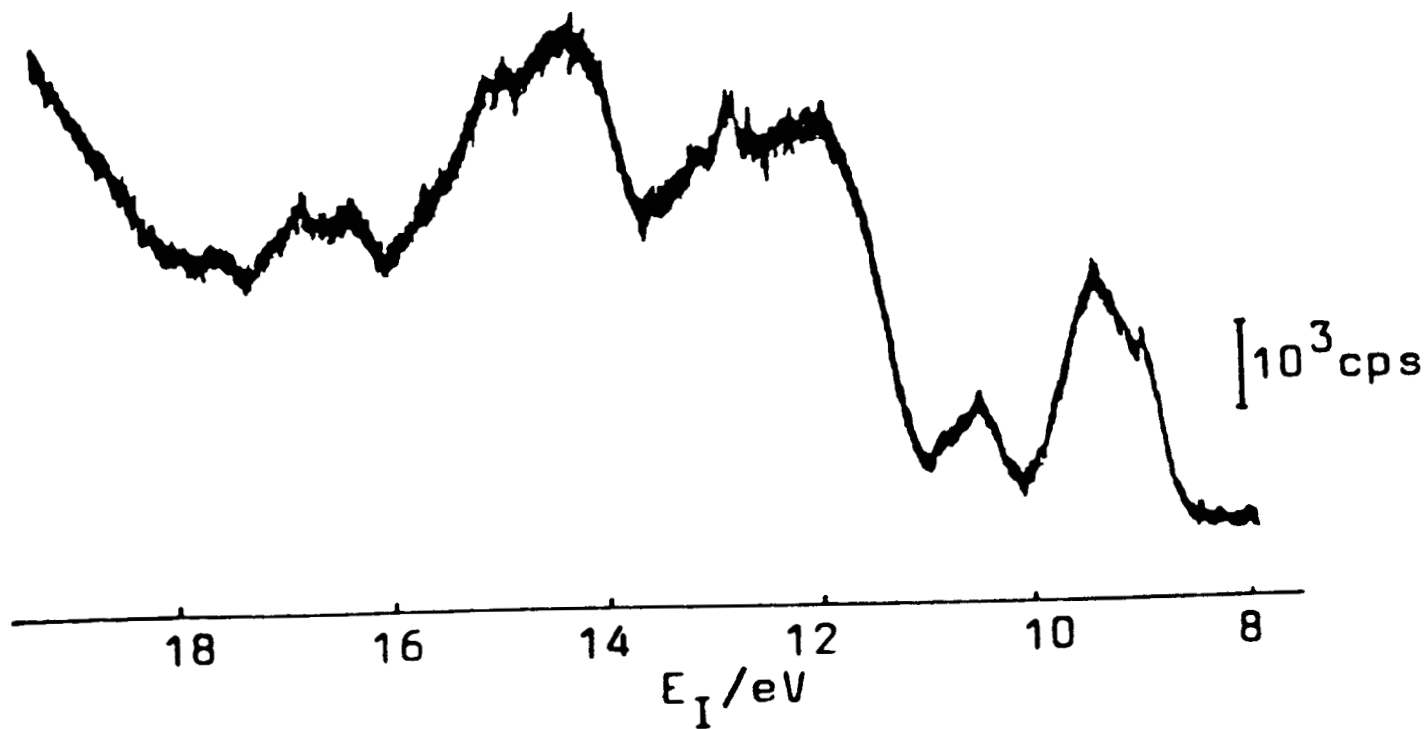


Figure 8 Photoelectronic Spectrum of Ketoprofen

2.12 Nuclear Magnetic Resonance Spectrum

The NMR spectrum of ketoprofen in CDCl_3 on a EM-360 60MHz. NMR spectrometer is given in Figure 9. Table 1 compares the published values for the NMR spectrum.

TABLE I

Instrument Used	Values	Ref
Varian EM-360	1.53(d,3H,J = 7Hz), 3.80(q,1H,J = 7Hz), 7.20-7.90(M, 9H), 11.50(s,1H)	
Varian T60 & A60	1.52(d,3H,J = 7Hz), 3.76(q,1H,J = 7Hz), 7.2-7.8(m,9H), 11.8(s,1H)	(14)
Varian T60 & A60	1.51 (d,3H,J = 7.4Hz), 3.82(q,1H,J = 7.4Hz), 7.2-8.0(m,9H), 11.55(s,1H)	(5)

2.13 Infrared Spectrum

The infrared spectrum on a Perkin Elmer 710B instrument of ketoprofen is reported in Figure 10. The major band assignments are given in Table II.

TABLE II

Band Position cm^{-1}	Assignment
3200-2500	O-H stretching
3020	C-H stretching of aromatic
2970,2930	C-H stretching of CH_3 group (assymetric) Masked by O-H stretching
2880	C-H stretching of CH_3 group (symmetrical) masked by O-H stretching
1695	C=O stretching of acid
1655	C=O stretching of ketone
1595,1580,1455	C=C stretching of aromatic ring
1440	C-H deformation of CH_3 (assymetrical)
1370	C-H deformation of CH_3 (symetrical)
860-690 (several bands)	C-H deformation of aromatic rings

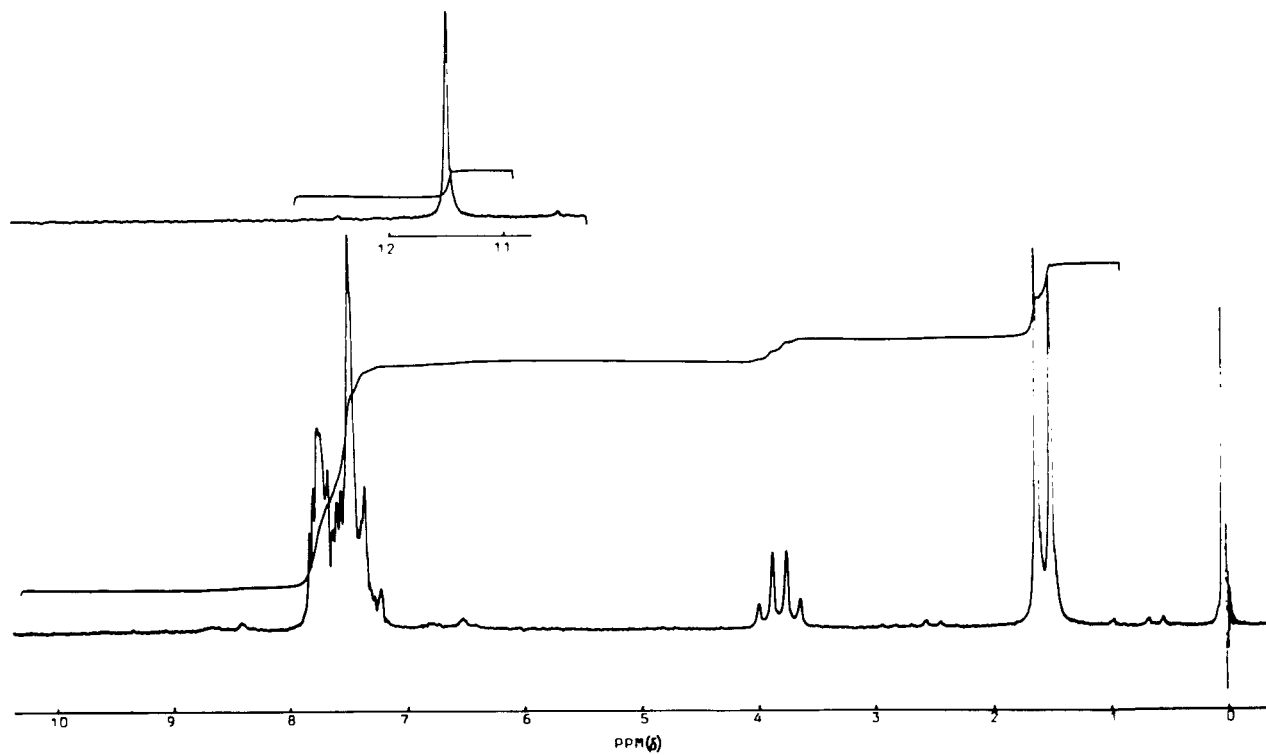


Figure 9 Nuclear Magnetic Resonance Spectrum of Ketoprofen

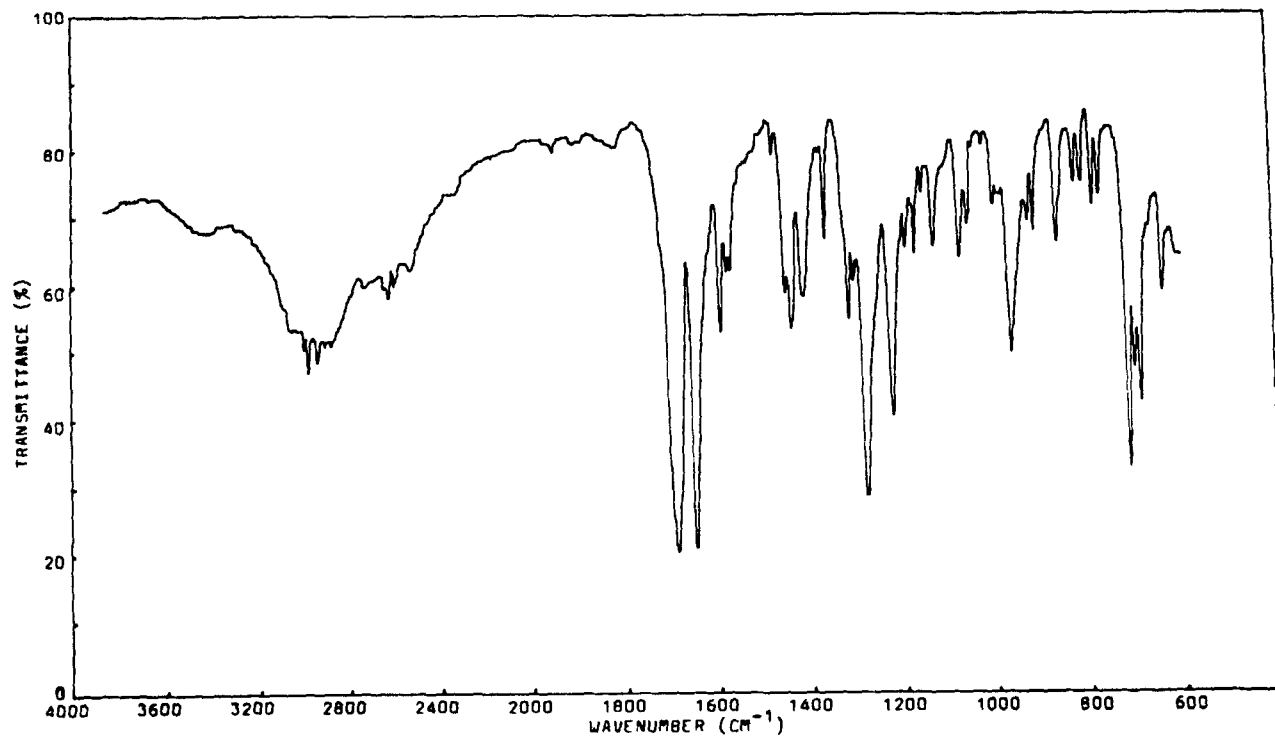


Figure 10 Infrared Spectrum of Ketoprofen

3. Synthesis

Several methods for the synthesis of ketoprofen have been reported in the literature (15-20), 23-27, 43, 46-54). The synthesis starting from (3-benzoylphenyl)-acetonitrile is illustrated in Figure 11 (15,16,41). The synthesis starting from (3-carboxyl-phenyl)-2 propionitrile is illustrated in Figure 12 (17,20). The synthesis starting from 2-(4-Aminophenyl) propionic acid is illustrated in Figure 13 (15,17,19).

4. Stability and Degradation

Ketoprofen must be protected from light and moisture. It is stable at room temperature. Ketoprofen has been dissolved in ethyl acetate and stored for several weeks at 4°C with no detectable decomposition (13). If ketoprofen is heated in an acid solution pH1 at 98°C for 30 min. no decomposition is detected (28).

5. Absorption, Metabolism and Excretion

Ketoprofen is absorbed rapidly regardless of the route of administration. It reaches a peak maximum in the first hour of administration if taken by the oral, rectal and parental routes and six hours if taken by the subcutaneous route. Peak blood levels by the rectal route are observed after 45 mins. to 60 mins. (29,30,31). Peak blood levels by the oral route are observed after 60 mins. to 90 mins. (31,32,33) and by the intramuscular route after 30 mins. (32). The half life of ketoprofen has been reported from 1.5 hrs. to 2 hrs. (6,29,30, 32-36). From 60% to 90% of ketoprofen is bound to serum protein (29). The kinetics of elimination are first order and the rate constant is 0.350 hrs., 63% of the administered dose is excreted in the urine during the first 24 hrs. and 65. in the first 48 hrs. Minimal excretion occurs in the faeces, the rat being the exception (29). The metabolism of ketoprofen is due to two major processes, a hydroxylation process, predominant in the rat, although the preferred excretory form in the rat is unchanged ketoprofen and glucuronide conjugation in other species including man. The glucuronide conjugation pathway is predominant in the rabbit and man but in man the hydroxylation is not totally absent

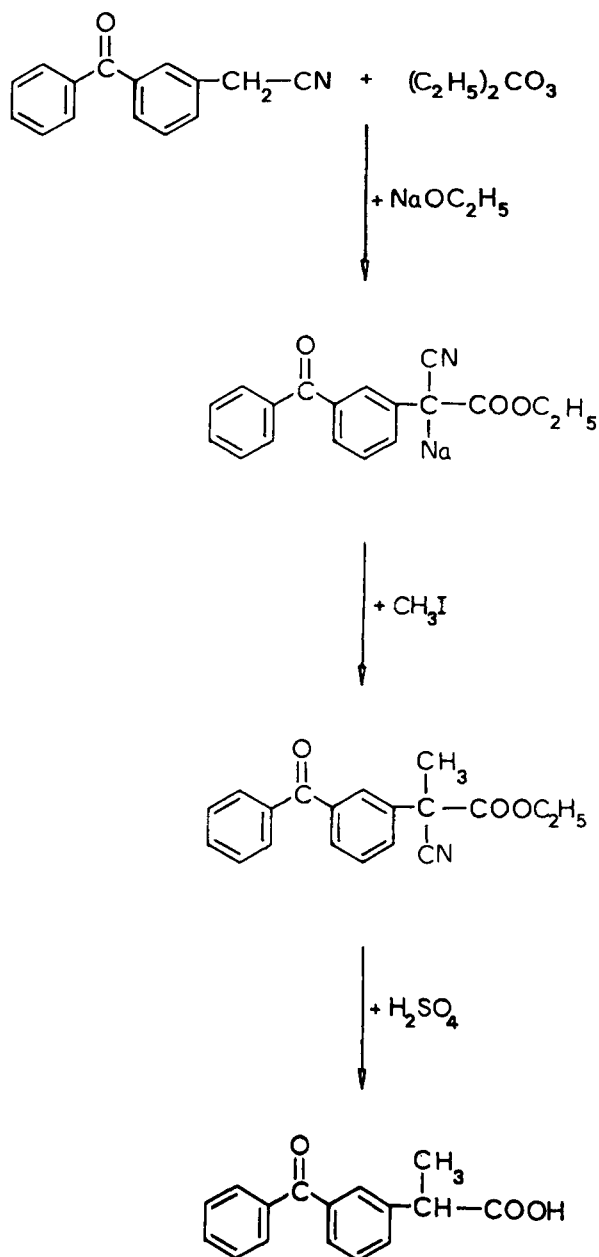


Figure 11 Synthesis of Ketoprofen Starting From (3-benzoylphenyl)-acetonitrile

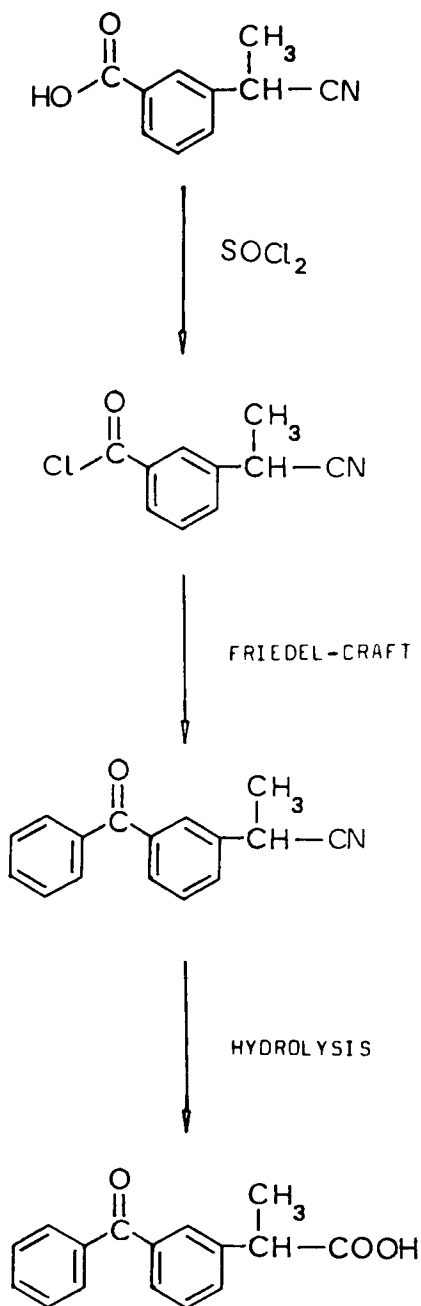


Figure 12 Synthesis of Ketoprofen Starting From (3-carboxy-phenyl)-2 propionitrile

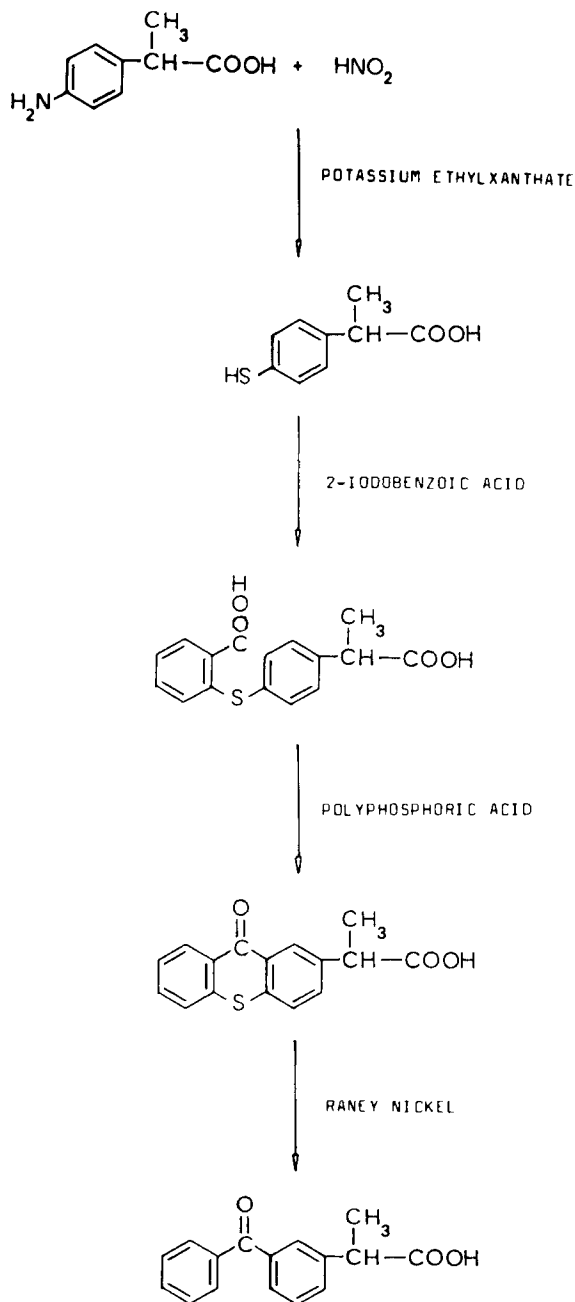


Figure 13 Synthesis of Ketoprofen Starting From 2-(4-Aminophenyl) propionic acid

see Figure 14 (29,31). As mentioned in section 2.9 Optical Rotation ketoprofen can exist in two enantiomeric forms, each having a different affinity for human serum albumin and different biotransformation pathways (Hydroxylation pathways) (4).

6. Methods of Analysis

6.1 Elemental Analysis

C(75.58%), H(5.55), O(18.87%)

6.2 Thin Layer Chromatographic Analysis

Thin layer chromatography on silica is suitable as a fast preliminary purity test. Table III gives the R_f values for ketoprofen.

*= R_x values relative to benzoic acid

TABLE III

Stationery phase and platecoating	Mobile phase	R_f (References)
Cellulose 0.10 cm thick	sec-butanol: absolute ethanol: water:ammonia 32% (50:30 19:1) v/v.	1.35 (21) *
Silica gel 0.25 cm thick	chloroform:methanol: ammonia 32% (120:60:0.5) v/v .	1.42 (21) *
Silica gel 0.25 cm thick	hexane:acetone:water (12.15:3) v/v.	0.72 (21) *
Silica gel 0.25 cm thick	iso-octanol:dioxan: glacial acetic acid (20:20:1) v/v.	0.88 (21) *
Silica gel 0.25 cm thick	butyl acetate:methyl ethyl ketone: glacial acetic acid:water (4:4:2:1) v/v	1.03 (21) *
Silica	chloroform:methanol (94:6)	0.42 (5)
Silica	n-hexane: dimethylketone: acetic acid (90:10:2)	0.05 (5)
Silica	dichloromethane: methanol:conc ammonia (70:30:3)	0.75 (5)

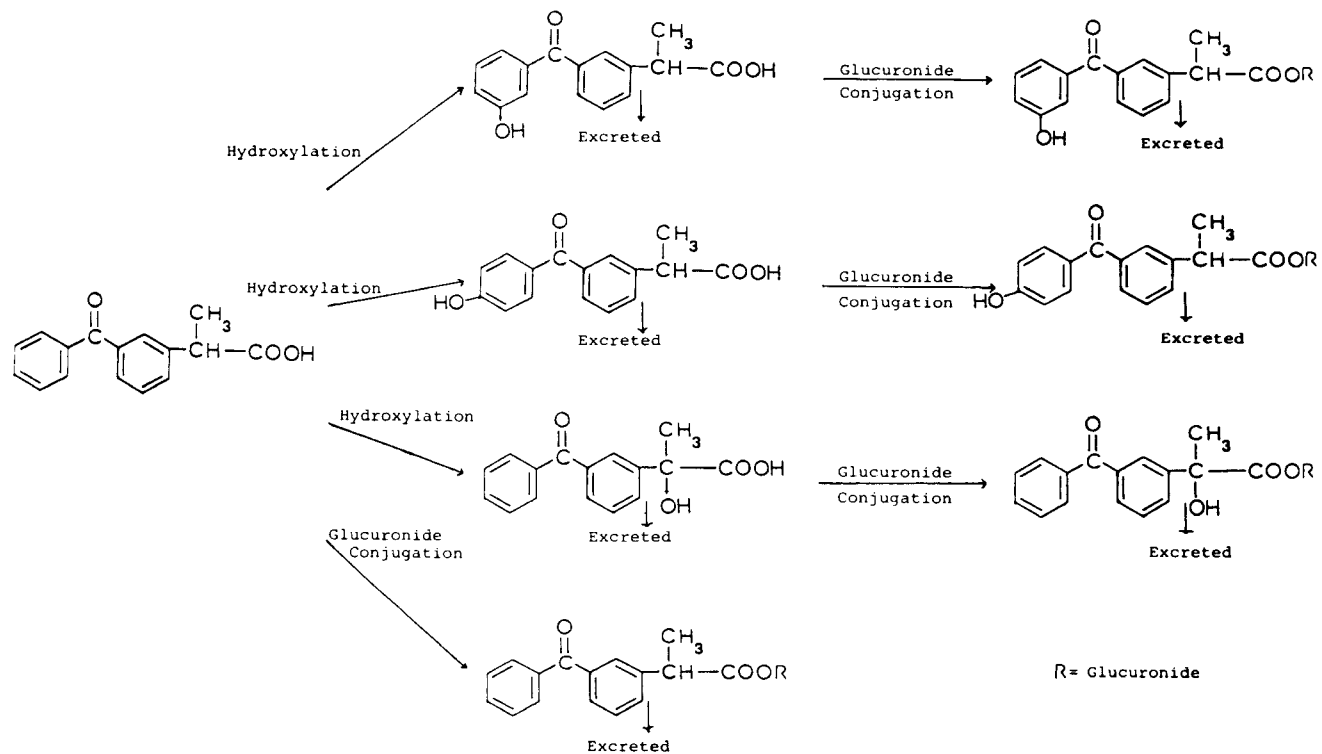


Figure 14 Metabolism of Ketoprofen

6.3 Ultra Violet Spectroscopy

Quantitative determinations of ketoprofen based on the peak maximum at 261 nm in distilled water (5) or 256 nm in methanol can be performed (21) see Section 2.8 and Figure 3.

6.4 Potentiometric titration

If ketoprofen is accurately weighed and dissolved in acetonitrile:water (3:1) and titrated with 0.1 NaOH the potentiometric curves are recorded between pH 3.45 - 12.0. This method is convenient for measuring the purity of ketoprofen in the crystalline preparation and also the content in tablets (5).

6.5 Gas Chromatography

Gas chromatography is an inconvenient method for purity determination, as ketoprofen is partially decomposed by the procedure. This can be overcome by using the methylester or trimethylsilylester prepared quantitatively from ketoprofen. According to the method of Populaire et al (37) ketoprofen and the esters can be chromatographed on a 1 m x 3 mm column of OV-17, 5% GCQ 80-100 mesh at a temperature between 230 - 270°C and carrier gases; argon:50 ml/min, hydrogen:79 ml/min, air:150 ml/min, and a Flame Ionisation Detector. The retention times R_t are for ketoprofen 6.35 min, for the methyl ester 4.05 min and for the trimethylsilylester 2.68 mins (5).

6.6 Enantiomer Analysis

The ratio of (+)-ketoprofen to (-)-ketoprofen in a racemic mixture can be determined by reaction with a stereospecific molecule and the product is then analysed by either gas chromatography (5) or high pressure liquid chromatography (38). The ratio of peak height or peak area respectively gives the ratio of the two enantiomers.

6.7 Colorimetric Analysis

Ketoprofen can be complexed with safranin and the absorption determined in chloroform at 520 nm (14).

7. Analysis of Biological Samples

7.1 Colorimetric Analysis

This method is suitable for the analysis of ketoprofen in urine. The urine is made alkali by addition of NaOH then extracted with ether, the aqueous layer is then acidified and the ketoprofen extracted with hexane and evaporated to dryness. The ketoprofen (via its carbonyl group) is reacted with p-nitrophenylhydrazine to give a p-nitrophenylhydrazone which gives a violet colour with trimethylbenzylammonium hydroxide. This violet complex is then assayed colorimetrically at 560 nm and 460 nm (see Figure 15 for spectrum of blank and sample —). The full experimental detail is reported by P. Populaire et al (37). The p-nitrophenylhydrazone formed undergoes partial decomposition to give other hydrazones, but these also absorb at 560 nm and are directly proportional to the concentration of ketoprofen in total and this decomposition does not interfere with the assay. The absorption due to ketoprofen at 560 nm is determined by subtraction of the absorption by urine blanks, at 460 nm ($460 \text{ nm Abs} = 560 \text{ nm Abs for blank}$) from it, this is the case for humans and cats. But for rats and rabbits the absorbance at 480 nm is deducted from the absorbance at 560 nm i.e. interference is species dependent. The precision of this method is $\pm 10\%$ over the urinary concentration range of 10-100 mg/L and the limits of detection of 2-5 mg/L in urine.

7.2 Polarographic Analysis

This method is suitable for the analysis of ketoprofen in urine. The same extraction procedure as in Section 7.1 is used as urinary substances will interfere with the polarogram. The carbonyl group of ketoprofen is reduced at the dropping mercury electrode, in a 0.2 M solution of

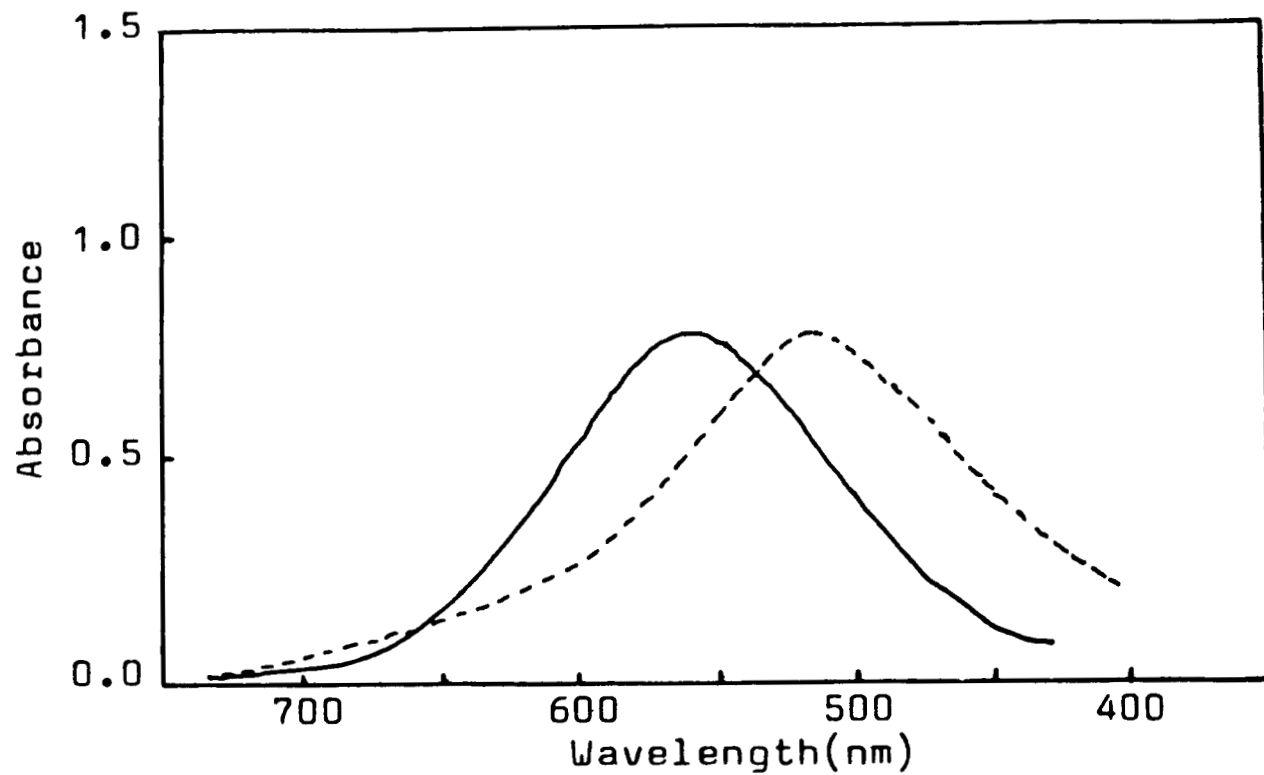


Figure 15 Colourmetric analysis of Ketoprofen in Biological Samples

tetrabutylammonium hydroxide. The half wave potential $E_{1/2}$ is -1.36 volts, see Figure 16 and the method employs a standard addition technique. For further experimental details see P. Populaire (37). The precision of this method is $\pm 10\%$ of urinary concentrations in the range 10 to 100 mg/litre and the limit of detection is 5 mg/litre.

7.3 Gas Chromatographic Analysis

If ketoprofen is analysed directly by gas chromatography partial decomposition results. This difficult can be overcome by working with the methyl ester (5,8,13,30,31,34,35,37,39), and using (Benzoyl-4-phenyl)-2 butyric acid as the internal standard as its methyl ester. Neither serum or urine samples of ketoprofen are gas chromatographed directly, an extraction procedure is employed. In the case of serum the sample is acidified then extracted by an organic solvent usually ether. In the case of urine the sample is made alkali and the unwanted products extracted with an organic solvent usually ether, the aqueous phase is then acidified and extracted with ether. The etherial extracts are washed with acid and then water, dried with magnesium sulphate and then evaporated to dryness. The samples are then methylated and chromatographed (5,29,30,31,34,35,37). In some cases further purification prior to gas chromatography is performed using thin layer chromatography (37). Table IV gives the conditions of chromatography and retention times (R_t) of the methyl esters of ketoprofen (A) and the internal standard (Benzoyl-4-phenyl)-2 butyric acid (B) obtained from biological media. The accuracy of this technique has been claimed as $\pm 10\%$ and a lower limit of detection of 0.03 - 0.04 mg/litre (37).

TABLE IV

Length and diameter internal (Reference)	Support, stationary phase and Temp.	Carrier gas and flow rate	Rt (A)	Rt (B)
6ft x 2mm (35)	OV-17 3% GCQ 80/100 mesh 250°C for 10 mins then 280°C for 4 mins.	N ₂ 60ml / min	6.29	8.85
1.5m x 2mm (8)	OV-1 3% GCQ 180°C-250°C at 2°C/min	He 20ml /min	14	18
2m x 2mm (13)	OV-1 1% GCP 100/120 mesh 250°C	He 30ml /min	3	5
2m x 3mm (29,39)	OV-17 1% Chromosorb W AW DMCS 240°C	N ₂ 30ml /min	4.8	6.6
1.5m x 2mm (10)	OV-17 3% GCQ 80/100 mesh 225°C	N ₂ 30ml /min	3	4.5
2m x 3mm (40)	OV-17 3% Chromasorb W AW DCMS 240°C	N ₂ 30ml /min	4.8	6.6
1m x 3mm (5)	OV-17 5% GCQ 80/100 mesh H ₂ O 230-270°C	Ar 50ml/min H ₂ 79ml/min air 150ml/min	4.05	

7.4 Thin Layer Chromatographic Analysis

A TLC method for the analysis of ketoprofen and its urinary metabolites has been described (31) using a two dimensional development system. But the separation is incomplete and the system insufficiently sensitive.

After extraction from biological samples as in Section 7.3 ketoprofen can be analysed by TLC, using 250 µm thick Merck 60 F254 plates, activated at 105°C for 1 hour, with a solvent system of ether-benzene-1-butanol-methanol (85:8:6:1), giving an R_f value of 0.75. The spot can be

scraped off the plate, dissolved in ethanol and analysed under UV. Accuracy of $\pm 6.01\%$ and a limit of detection of $1 \mu\text{g}$ is claimed by Ballerini et al (7). A further method involving methylation of ketoprofen has been described (37).

7.5 Gas Chromatography - Mass Spectroscopy Analysis

Ketoprofen in biological extracts is converted to methyl ester with an internal standard (benzoyl-4-phenyl)-2-butyric acid before undergoing GC-MS, as ketoprofen undergoes rearrangement when subjected to gas chromatography, see Section 6.5 (8,10,13). The limits of detection by this method are 2.5 ng and an accuracy of 10% at plasma concentrations of 25 ng/ml (8).

7.6 High Pressure Liquid Chromatographic Analysis

Most methods for the analysis of ketoprofen in biological samples require the selective extraction of ketoprofen (42) similar to Section 7.3 (12,28,39-42), or by direct injection (44). Table V gives the chromatographic conditions retention times (Rt), limit of detection and accuracy for ketoprofen.

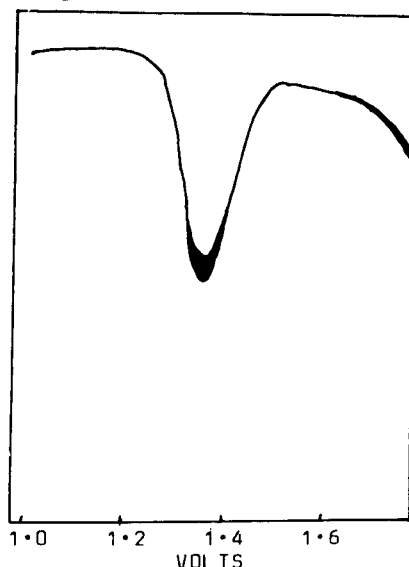


Figure 16 Polarogram of Ketoprofen

TABLE V

Column, Packing, diameter internal (Reference)	Mobile phase, flow rate, pressure	Rt min	Detection limit and accuracy
Hewlett Packard RP8-79918 A 250x4mm (7)	Water/Methanol 85/15, 0.8 ml/min 32-38 atmos	8.3	0.1 µg/ml ±5.1%
Spherisorb-5 ODS 50 x 5 mm (44)	35% aqueous methanol pH3 2 ml/min	2	2.5 ng ±2.1%
Lichrosorb S1 60 Sµm 250 x 4.7 mm (39,40)	dichloromethane/ hexane 60/40 1.3 ml/min 35 bars	*12.8	0.1 µg/ml ±4%
Lichrosorb RP 18 Sµm 150 x 4.7 mm (39).	methanol/ acetonitrile/ phosphate 15/35/45 pH3 0.83 ml/min 70 bars	8	0.1 µg/ml ±4%
Sphenisorb 5 µm C ₈ 40 x 4.6 mm (42)	0.5 phosphate pH 7.0 16-8% acetonitrile 2.0 ml/min 1000 psi	16	10-20 ng i ml
µ Bondapak C ₁₈ 300 x 4 mm (12)	methanol/water 45/44 1.1 ml/min 1800 p i	5.3	0.1 µg/ml ±5.4%
Lichrosorb RP 18 5 µm or Lichrosorb RP 8 5 µm. 100 x 4.6 mm (9)	acetonitrile/ phosphate 0.02 m pH3 45:55 1000 psi	5.2 4.2	} 0.04 µg/ml ±4%

* methyl ester of ketoprofen

8. Analysis of Pharmaceutical Formulations

8.1 Potentiometric Titration

300 mg of ground sample are dissolved in 5 mls of acetonitrile or ethanol and 15 ml of water added. The titration is performed with 0.1 N NaOH

and monitored using a glass electrode and a calomel reference electrode (5).

8.2 Pyrolysis - Gas Chromatography - Mass Spectrometry

Ground samples are dissolved in a solvent and a known amount applied to a rotating wire. After evaporation of the solvent the material is pyrolysed in a Curie point pyrolyser at 770°C for 5 seconds and the pyrolyate purged onto a Carbowax 20 M - KOH column, that is temperature programmed from 100 to 240°C. On pyrolysis ketoprofen is rearranged to (3-benzoylphenyl)-ethane and (3-benzoylphenyl)-ethylene which have retention indices of 2.27 and 2.52 respectively, analine having a retention of 1.00. Limits of reproducible detection range from 10 ng to 10 µg. This method can also be employed in the analysis of biological materials (45,22).

9. Acknowledgements

I wish to express my thanks to Mrs. T. Bowler for typing this manuscript.

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METHYLPHENIDATE HYDROCHLORIDE

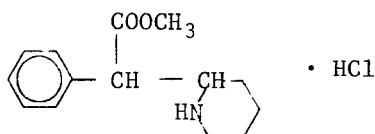
Gandharva R. Padmanabhan

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1. Description

1.1. Name, Formula, Molecular Weight

Methylphenidate hydrochloride is methyl α -phenyl-2-piperidineacetate hydrochloride, (R*,R*)-(±).



$C_{14}H_{19}NO_2 \cdot HCl$

Molecular Weight 269.77

1.2. Appearance

Methylphenidate hydrochloride occurs as a white, odorless, fine, crystalline powder.

2. Physical and Chemical Properties

2.1. Infrared Absorption Spectrum

The infrared spectrum of a mineral oil suspension of methylphenidate hydrochloride is shown in Figure 1. The spectral assignments are listed in Table 1.

TABLE 1

Wavenumber, cm^{-1}	Assignment
703, 737	Monosubstituted benzene
1602	Aromatic Stretch
2300 - 2700	Secondary Amine Salt
1739	C=O Stretch
1150 - 1170	C-O Stretch

2.2. Nuclear Magnetic Resonance Spectrum (NMR)

The NMR spectrum of methylphenidate hydrochloride is shown in Figure 2. The spectrum was determined on a Perkin-Elmer R-24B 60 MHz spectrometer at ambient temperature. The sample was dissolved in a 1:1 mixture of deuterated chloroform and deuterated dimethylsulfoxide containing tetramethylsilane as an internal standard. The spectral assignments are shown in Table 2.

Figure 1:
Infrared Absorption Spectrum of Methylphenidate Hydrochloride

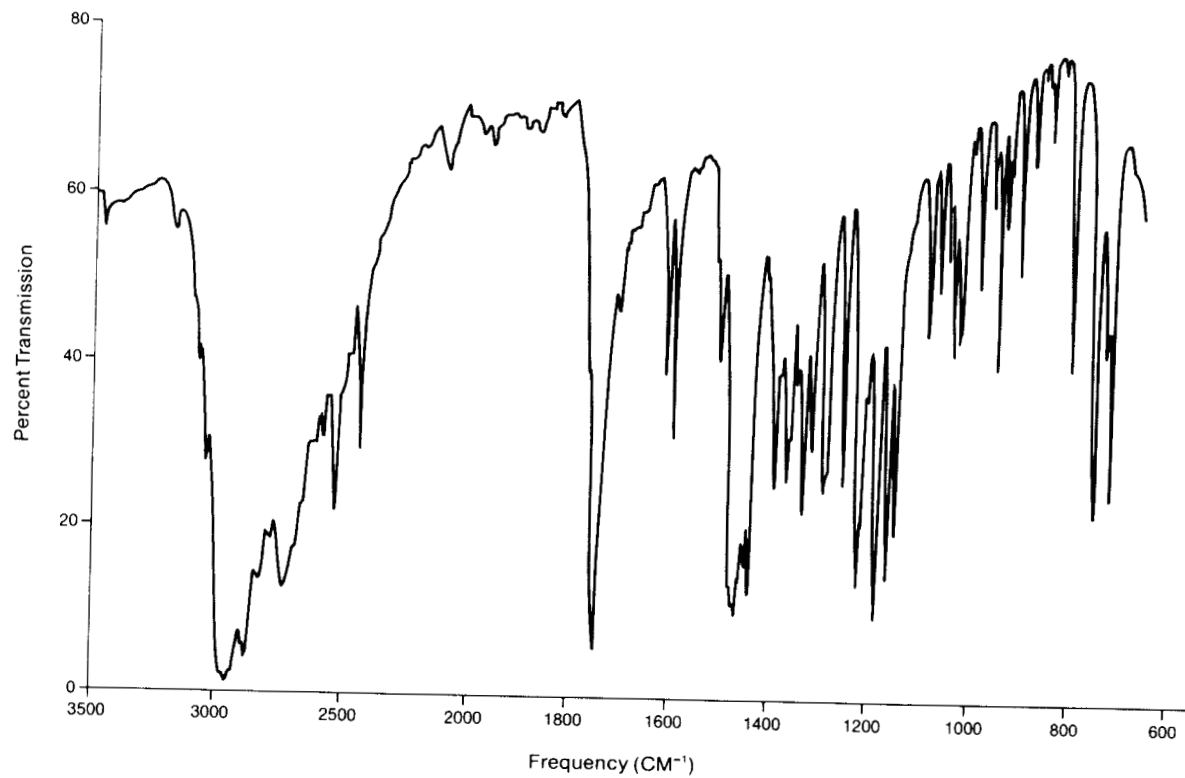
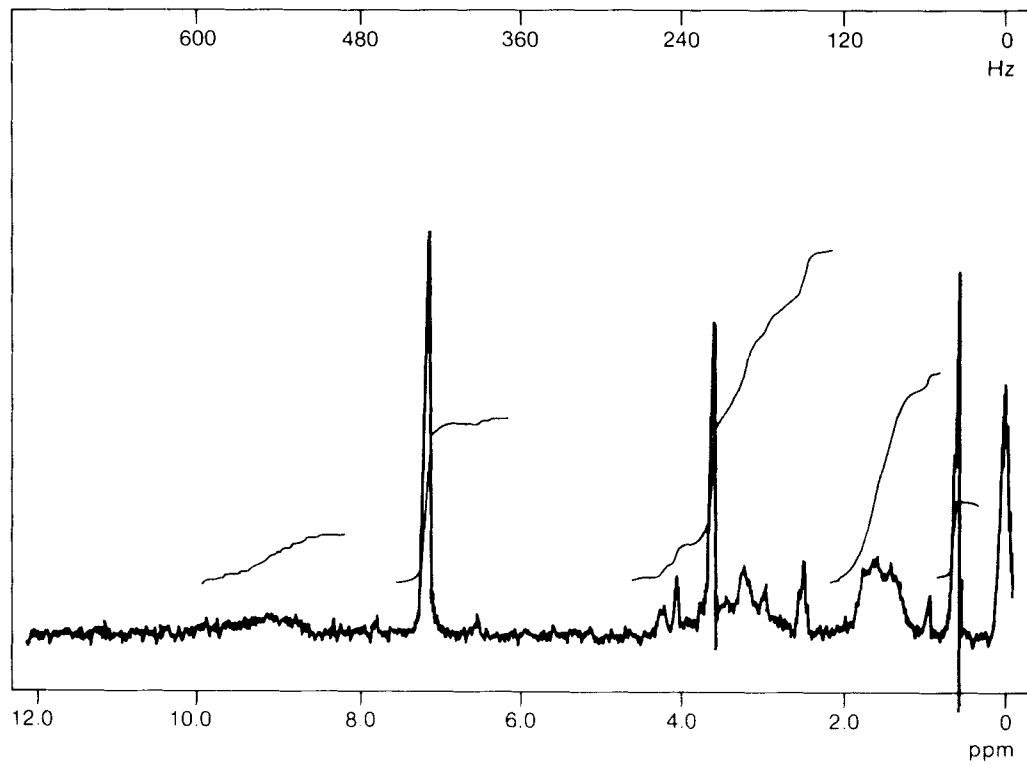


Figure 2:
NMR Spectrum of Methylphenidate Hydrochloride



Perkin-Elmer R-24B 60MHz NMR Spectrum

TABLE 2

Chemical Shift δ (ppm)	Multiplicity	No. of Protons	Assignment
7.1 - 7.6	Broad Singlet	5	Phenyl protons
4.0 - 4.4	Doublet	1	$\begin{array}{c} \\ -\text{CH}-\text{COOCH}_3 \end{array}$
3.6 - 3.8	Singlet	3	$-\text{OCH}_3$
2.8 - 3.6	Broad Multiplet	3	$\begin{array}{c} \\ \text{CH}- \\ \\ -\text{N} \begin{array}{l} \text{CH}_2- \\ \text{CH}_2- \end{array} \end{array}$
2.4 - 2.7	Broad Multiplet	-	Solvent
1.0 - 1.9	Broad Multiplet	6	$-\text{CH}_2-\text{CH}_2-\text{CH}_2-$

2.3. Ultraviolet Absorption Spectrum

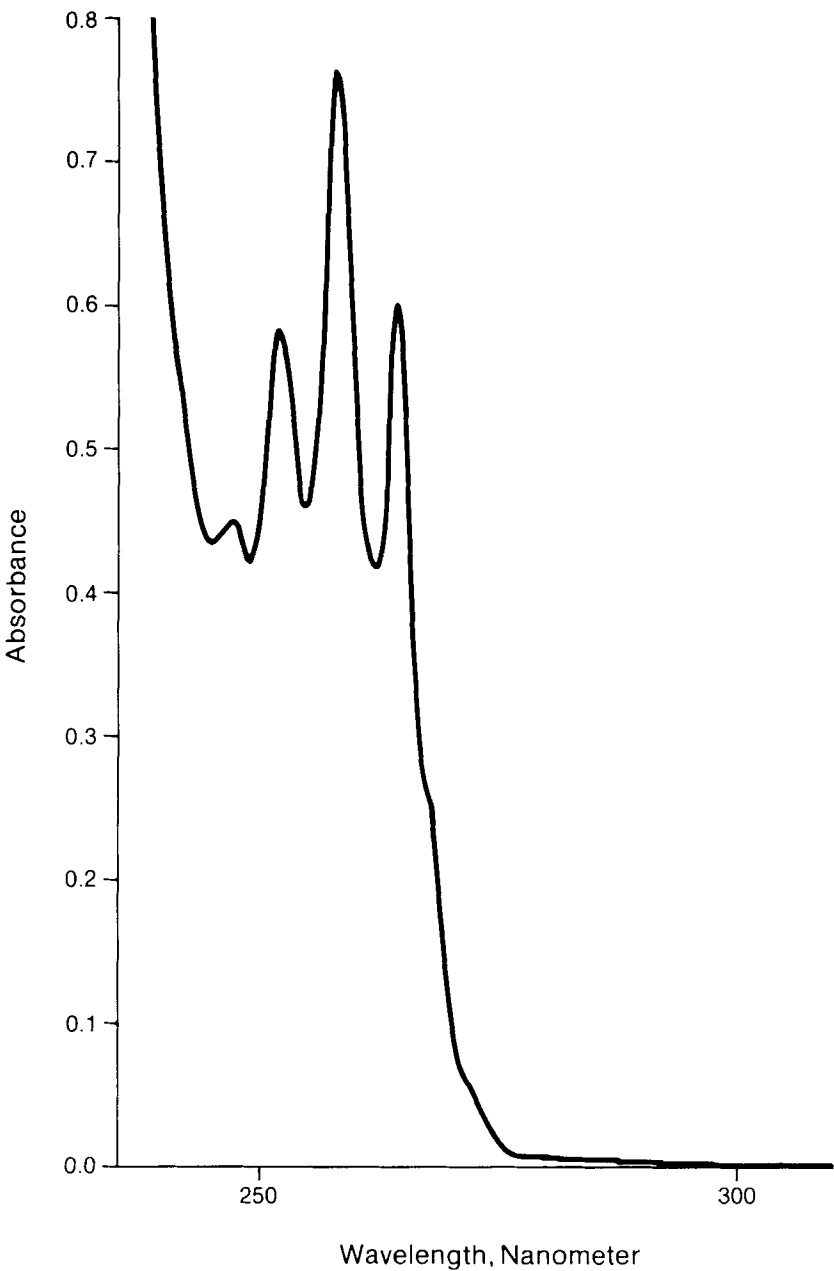
The UV spectrum of methylphenidate hydrochloride (1 mg/mL) in methanolic 0.1N HCl exhibits maxima and minima as shown in Table 3 and Figure 3.

TABLE 3

λ max, nm	$A_{1\text{cm}}^{1\%}$	ϵ
264	6.1	165
257	7.7	208
252	5.9	159
247	4.5	122

λ min. at 263 nm, 255 nm, 249 nm and 245 nm.

Figure 3:
Ultraviolet Absorption Spectrum of Methylphenidate Hydrochloride



2.4 Mass Spectrum

The low resolution mass spectrum of methylphenidate hydrochloride obtained at 70 ev using a solid probe insertion is shown in Figure 4. The spectrum was run on a Kratos MS25 spectrometer interfaced with a data handling system. Table 4 illustrates the prominent fragments and their mass/charge ratios.

TABLE 4

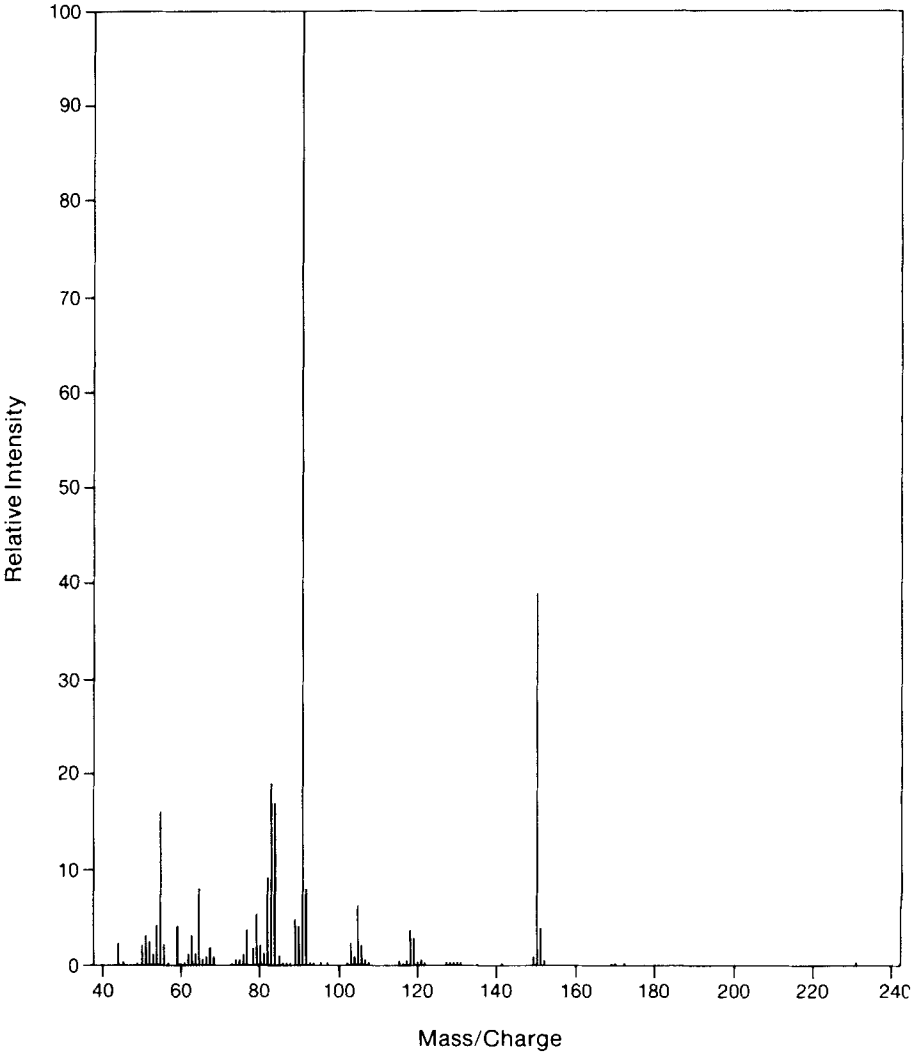
m/e	Fragment*
232, 234	$[M - H]^+$ and $[M + H]^+$
174	$[M - \text{COOCH}_3]^+$
150	$[234 - \text{C}_6\text{H}_5\text{CH}_2]^+$
91	$\text{C}_6\text{H}_5\text{CH}_2^+$
84	$[\text{C}_6\text{H}_5\text{CH}_2\text{N}^+ \text{C}_5\text{H}_4]^+$
56	$[84 - \text{C}_2\text{H}_4]^+$

*The spectrum is known to vary due to thermal decomposition (27).

2.5 Optical Rotation

Although the methylphenidate hydrochloride molecule has two asymmetric carbon atoms, the drug exhibits no optical activity as it is a racemic mixture. The diastereoisomer of the drug, (R^*, S^*) isomer, is also referred to as "erythro isomer". The conformations of methylphenidate hydrochloride and its (R^*, S^*) isomer have been documented (1-2). Due to the low efficacy of the (R^*, S^*) isomer (1) the amount of this diastereoisomer is controlled in the drug to a level of 1% or less (3).

Figure 4:
Low Resolution Mass Spectrum of Methylphenidate Hydrochloride



2.6 Melting Range

Methylphenidate hydrochloride melts between 224°C and 226°C when tested according to the USP XX Class Ia procedure.

2.7 Differential Scanning Calorimetry (DSC)

The DSC thermogram of methylphenidate hydrochloride shows a melt endotherm between 195°C and 235°C with a melting point, with decomposition, of approximately 220°C when the thermogram was followed in a DuPont Model 900 instrument at a scan rate of 10°C/minute (Figure 5).

2.8 Thermogravimetric Analysis (TGA)

The TGA of methylphenidate hydrochloride exhibited a weight loss of 0.34% between 30°C and 150°C. Above 150°C a rapid weight loss due to decomposition and/or sublimation was observed.

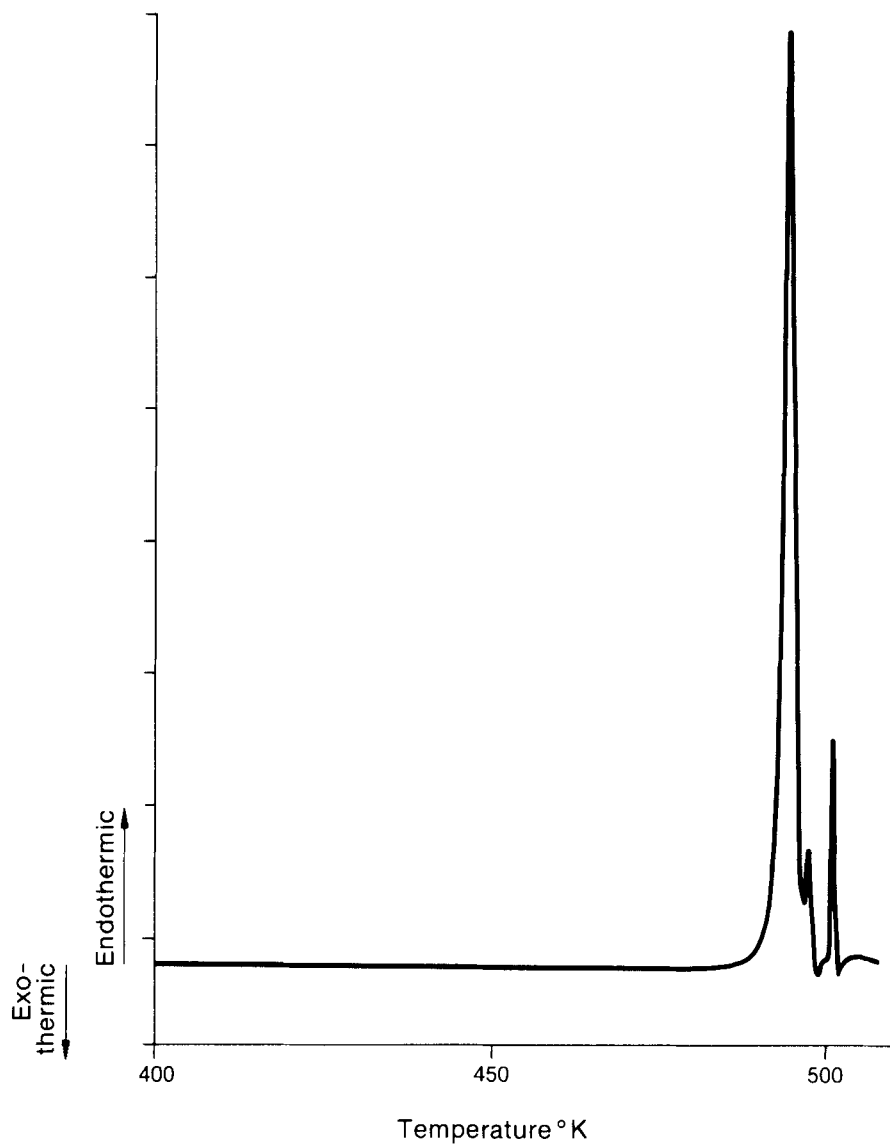
2.9 Solubility

Approximate solubilities in different solvents were determined after equilibrating 10 mg (more, if necessary, to obtain a saturated solution) of the drug at room temperature with 1 mL of solvent.

TABLE 5

Solvent	Solubility (mg/mL)
Water	> 100
0.1N HCl	> 100
Methanol	> 100
Ethanol	> 25
Acetonitrile	5.3
Chloroform	> 100
n-Hexane	< 0.01
Ethyl Acetate	0.08
Ether	< 0.01
95% Ethanol	> 50
Petroleum Ether	< 0.01
Acetone	0.9

Figure 5:
DSC Scan of Methylphenidate Hydrochloride



2.10 X-Ray Diffraction

The x-ray powder diffraction pattern obtained for methylphenidate hydrochloride is shown in Figure 6. The data were collected on a GE Model XRD-spectrogoniometer using Cu K_α (1.542 Å) with a Ni filter as a radiation source.

2.11 Polymorphism

No polymorphism has been reported for methylphenidate hydrochloride.

2.12 Partition Coefficient

The following partition coefficient data were obtained when 50 mL of 0.1 and 1.0 mg/mL of methylphenidate hydrochloride in appropriate aqueous solutions at room temperature were partitioned individually with 50 mL of indicated organic solvents. Heptane-pH 7.4 buffer data was obtained from the literature (4).

TABLE 6

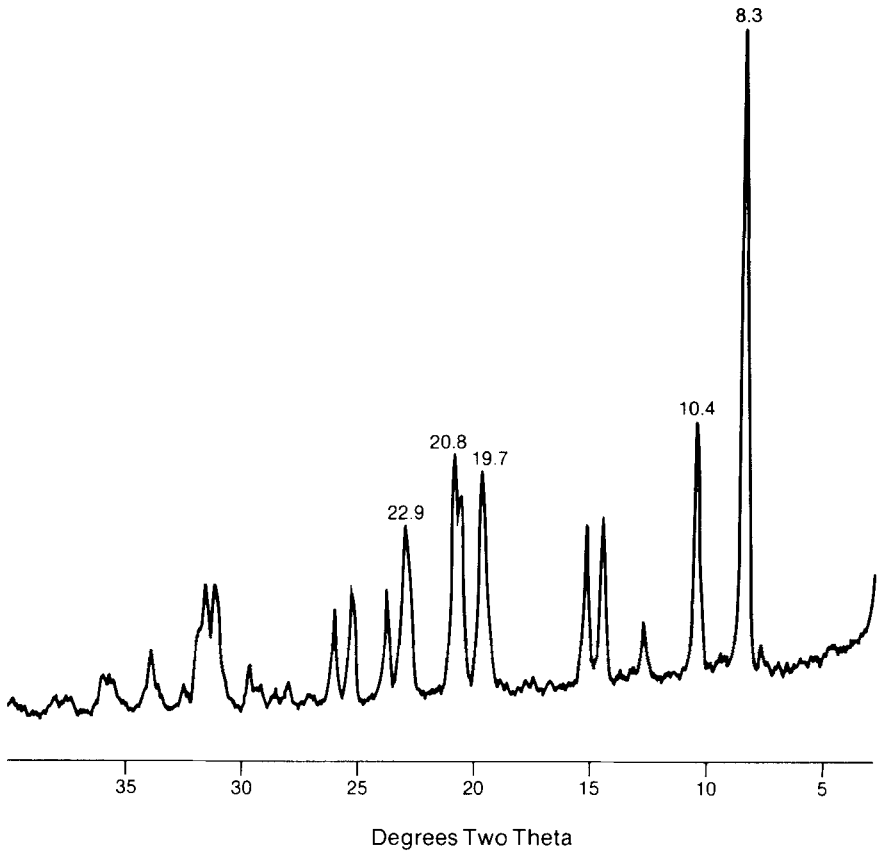
Aqueous Phase	Organic Phase	Partition Coefficient*
0.1N HCl	Chloroform	$\rightarrow 0$
pH 7 Buffer	Chloroform	22.7 ± 0.5
0.1N HCl	Ether	$\rightarrow 0$
pH 7 Buffer	Ether	1.7 ± 0.2
pH 7.4 Buffer	Heptane	0.63

* = Concentration in organic phase/concentration in aqueous phase.

2.13 Dissociation Constant

A pKa value of 9.0 was obtained for the dissociation of the protonated secondary amine function by potentiometric titration method. A value of 8.9 was also obtained (5) for the pKa by the non-logarithmic method of Benet and Goyan (6).

Figure 6:
X-Ray Powder Diffraction Pattern of Methylphenidate Hydrochloride



3. Synthesis

Methylphenidate hydrochloride is prepared by the following sequence of reactions. α -Phenyl-2-pyridineacetonitrile is hydrolyzed in diluted sulfuric acid to α -phenyl-2-pyridineacetamide. The acetamide is isolated and then hydrogenated over a catalyst to yield racemates of diastereoisomeric mixtures of α -phenyl-2-piperidineacetamide. The diastereoisomeric piperidineacetamide racemate mixture is converted to (R^* , R^*) racemate by heating in sodium hydroxide solution and then to α -phenyl-2-piperidineacetic acid by heating in sulfuric acid solution. The acetic acid derivative is converted to the acid chloride and then, without isolation, reacted with methanol to yield the methyl ester free base which is then converted to methylphenidate hydrochloride (7).

4. Stability-Degradation

The drug is relatively stable in acidic solutions but is degraded extensively in basic solutions. The degradation occurs via the hydrolysis of the methyl ester to the free acid, α -phenyl-2-piperidineacetic acid (8-10).

<u>pH of Solution</u>	<u>Temperature °C</u>	<u>Time (hours)</u>	<u>% Methylphenidate HCl Remaining</u>
¹ 1.7	100	20	100
² 3.7	100	20	100
² 3.5	100	300	95
² 4.9	100	20	84
² 4.1	100	300	51
³ 5.7	100	20	49
⁴ 8.9	100	20	0
⁴ 9.2	30	20	87

¹0.1N HCl

²Phthalate Buffer

³Phosphate Buffer

⁴Borate Buffer

The methyl ester of phenylacetic acid has been reported as one of the products when the drug substance was subjected to thermal degradation.

5. Drug Metabolism and Pharmacokinetics

α -Phenyl-2-piperidineacetic acid, the lactam acid and several other unidentified polar compounds have been reported as metabolites in guinea pig, dog and human urine (4, 11-13). p-Hydroxyphenyl-2-piperidineacetic acid and its methyl ester were also reported as metabolites in dog and rat urine (14). The products of metabolism involving oxidation, hydrolysis and conjugation processes in rats and dogs have also been reported (30).

6. Toxicity

A typical sample of methylphenidate hydrochloride active drug gave an oral LD₅₀ value of 350 mg/kg with deaths (9/10) at 1000 mg/kg when a 7.5% solution was administered to male rats (15).

7. Methods of Analysis

7.1 Identification

Two identity tests are given in the USP XX, one an infrared absorption test and the other a test for chloride.

7.2 Elemental Analysis

The following elemental composition was obtained for methylphenidate hydrochloride when a 2 mg sample was employed for analysis with a Perkin-Elmer, Model 240 CHN Analyzer.

<u>Element</u>	<u>Theory, %</u>	<u>Found, %</u>
Carbon	62.33	62.35
Hydrogen	7.47	7.57
Nitrogen	5.19	5.12

7.3 Nonaqueous Titration

Methylphenidate hydrochloride may be titrated in glacial acetic acid containing mercuric acetate with perchloric acid in glacial acetic acid as titrant. The titration can be carried out potentiometrically or with p-naphtholbenzein as indicator.

Although the titration as such is not specific for the intact drug in presence of its major degradation compound, α -phenyl-2-piperidineacetic acid, the procedure can be made specific by extracting the drug from a pH 9 buffer with chloroform and then titrating the free base with perchloric acid without the addition of mercuric acetate (16).

7.4 Phase Solubility Analysis

Phase solubility analysis of methylphenidate hydrochloride has been carried out using the following systems:

System I

Solvent: A mixture of 93 mL of benzene and
7 mL of anhydrous ethanol

Approximate solubility: 14 mg/mL

System II

Solvent: A mixture of 90 mL of benzene and
10 mL of methanol

Approximate solubility: 65 mg/mL at 30°C

System III

Solvent: n-Butanol

Approximate solubility: 13 mg/mL at 30°C

7.5 Thin-layer Chromatography

A number of thin-layer chromatographic systems have been developed for the identification and the determination of the drug and compounds related to the drug.

System I

The following system may be employed particularly to control the (R^* , S^*) isomer content in the drug (3).

Adsorbent: Silica Gel G plate, 250 μ thickness

Mobile Phase: A mixture containing 190 mL of chloroform, 10 mL of methanol and 1 mL of concentrated ammonium hydroxide.

Detection System: Dragendorff spray reagent (0.7 g of bismuth subnitrate dissolved in 40 mL of 20% glacial acetic acid and then diluted successively with 40 mL of 40% KI solution, 120 mL of glacial acetic acid and 250 mL of water).

<u>System IX</u>	Chloroform/Methanol (1:1); Silica Gel GF; Iodine Spray and UV Detection Systems (17).
<u>System X</u>	Chloroform/Methanol/Concentrated Ammonium Hydroxide (9:1:1); Silica Gel GF; Acetic acid-Commercial Chlorine Bleach-Phenothiazine Spray Reagent and Shortwave UV Detection Systems (20).
<u>System XI</u>	Chloroform/Methanol/Concentrated ammonium Hydroxide (7:5:1); Silica Gel GF; Detection Systems Same as in System X (20).
<u>System XII</u>	Acetone/Methanol containing 0.5% HCl (1:1); Silica Gel GF: Detection Systems same as in System X (20).
<u>System XIII</u>	Concentrated Ammonium Hydroxide/Ethanol/Water (16:100:12); Silica Gel GF; Detection System not reported.
<u>System XIV</u>	Methanol/Formic Acid (9:1); Silica Gel GF: Detection System not reported.
<u>System XVI</u>	Ethyl Acetate/Acetic Acid/Water/Hydrochloric Acid (55:35:2:2); Silica Gel G; Detection System not reported.

7.6 High Pressure Liquid Chromatography

The following two systems have been reported (21) for the quantitation of the (R*,S*) isomer in methylphenidate hydrochloride samples.

System I

Mobile Phase:	A mixture containing 85 mL of chloroform, 13.5 mL of cyclohexane, 1.5 mL of ethanol, and 0.5 mL of concentrated ammonium hydroxide.
Column:	100 cm x 2.1 mm steel column dry-packed with Sil-X® (Perkin-Elmer).
Detection:	UV (254 nm)
Temperature:	Ambient

System II

Mobile Phase: A mixture containing 80 mL of chloroform, 20 mL of cyclohexane and 1.5 mL of ethanol.

Column: 50 cm x 2.1 mm (i.d.) MicroPak-SI-10 (Varian)

Detection: UV (254 nm)

Sample: Inject 20 μ L of isolated free base in chloroform

Temperature: Ambient

The following system has been employed for the quantitation of methylphenidate in serum samples (22).

System III

Mobile Phase: pH 3.5 Phosphate buffer/high purity acetonitrile (80:20).

Column: μ Bondapak C₁₈ (Waters) (4 mm x 30 cm)

Temperature: 40°C

Flow Rate: 1.6 mL/minute

Detection: UV (192 nm)

Internal Standard: 4,5-Diphenylimidazole

The following system has been employed for the determination of α -phenyl-piperidineacetic acid in serum samples (23).

System IV

Mobile Phase: pH 3.8 Phosphate buffer/acetonitrile (83:7)

Column: μ Bondapak C₁₈, (Waters) (4 mm x 30 cm)

Temperature: 40°C

Flow Rate: 2.0 mL/minute

Detection: UV (192 nm)

Internal Standard: α,α -Dimethyl- β -methylsuccinimide

7.7 Gas Chromatography

The following system has been employed for the analysis of the drug substance in plasma.

System I

Column: 8 ft x 4 mm i.d. with 5% XE-60 on Gas Chrom Q (100 - 120 mesh)
Temperature: Column - 150°C; Detector - 185°; Injector - 185°
Detector: Flame ionization
Carrier: Nitrogen 70 cc/minute
Sample: Isolated free base dissolved in DMF

The following system (19) has been employed for identification of the drug in blood and urine samples.

System II

Column: 5 ft x ¼" O.D. (3mm i.d.) glass column, 2% Hi Eff 3A (Applied Science) on Gas Chrom Q (100 - 120 mesh).
Temperature: Column - 140°C; Detector - not reported; Injector - 220°C
Detector: Flame Ionization
Carrier: Nitrogen at 75 mL/minute

The following system has been employed for the analysis of several drugs including methylphenidate hydrochloride.

System III

Column: 8 ft x 1/8" glass column with 15% XF-1112 on Chromosorb X-HMDS
Temperature: Injector - 200°C; Column - Program 60° - 180° at 4°/minute; Detector - not reported.
Detector: Flame ionization
Carrier: Nitrogen 25 mL/minute

The following system has been employed to quantitate (R*,S*) isomer in methylphenidate hydrochloride samples (25).

System IV

Column: 200 cm x 2 mm i.d. glass column with 3% OV-225 on Gas Chrom Q (80 - 100 mesh).

Carrier Gas: Nitrogen 30 mL/minute

Detector: Flame ionization detector

Temperature: Column - 125°C; Detector - 200°C;
Injector - 200°C

Sample: Isolated free base in chloroform.

The following system was employed for the identification of the drug in a tablet formulation.

System V

Column: 8' x 4 mm i.d. with 5% XE-60 on Gas Chrom S (100 - 120 mesh)

Temperature: Column - 130°C; Injector - 158°C;
Flame Ionization Detector - 175°C

Carrier Gas: Nitrogen 60 cc/minute

Sample: Isolated free base in dimethylformamide

7.8 Gas Chromatography - Mass Spectrometry (GC-MS)

Sensitive methods for the analysis of methylphenidate hydrochloride and its hydrolytic degradation compound have been reported using GC-MS with selected ion monitoring for separation and detection. The following experimental conditions were used for the analysis of the drug and its metabolite in blood and urine samples.

Method I (27)

Column: 6 ft x 2 mm i.d. glass column containing 3% OV-1 on Gas Chrom-Q (100 - 120 mesh)

Detection: GC-MS selected ion monitoring m/e=91

Method I (27) (Continued)

Temperature: Injector, detector, capillary restrictor, separator and line of sight probe - 225°C; Column - 165°C; MS Electron Impact Source - 175°C.

Carrier: Helium 40 mL/minute

MS EI Source: 70 ev

Method II (28)

Column: 0.9 m x 2 mm i.d. glass column with 3% OV-17 on Gas Chrom Q

Detection: GC-MS Selected Ion Monitoring m/e=180

Temperature: Injector - 250°C; Column - 170°C; Membrane Separator - 170°C; Transfer line - 250°C

Carrier Gas: Helium 35 mL/min

MS EI Source: 70 ev

Sample: Derivatized with trifluoroacetic anhydride

Internal Standard: Ethyl ester analog of methylphenidate HCl.

7.9 Colorimetric Methods

7.9.1 Picrate Method

Methylphenidate hydrochloride can be assayed by the picrate ion-pair extraction method (3). The sample is extracted into a diluted sulfuric acid solution, mixed with a picric acid solution, pH adjusted to 5.0 and then extracted as a picrate ion-pair with chloroform. The extracted drug is quantitated colorimetrically by measuring absorbance at 405 nm. The method is specific for the drug in presence of its hydrolytic degradation compound.

7.9.2 Bromcresol Purple

Methylphenidate hydrochloride also forms a colored complex with bromcresol purple at pH 5.0. The complex can be extracted with chloroform and the content of the drug quantitated by measuring the absorption of the acidified extract at 420 nm. The hydrolytic degradation compound does not interfere.

7.9.3 Hydroxamic Acid

In basic solution, methylphenidate forms a hydroxamic acid with hydroxylamine (29). This hydroxamic acid forms a red complex with ferric ion in acidic solutions which can be quantitated colorimetrically by measuring the absorbance at 500 nm. The degradation compound does not interfere with the assay. However, the method is subject to interference from excipients in drug formulations such as lactose.

7.9.4 1,2-Naphthaquinone Sulfonic Acid

An automated procedure has been reported for the determination of methylphenidate hydrochloride in tablet formulations based on the formation of a yellow-colored complex with 1,2-naphthaquinone sulfonic acid. The complex is extracted into chloroform and the drug quantitated by the measurement of absorption maximum of the complex at 400 nm.

7.10 Infrared

Methylphenidate hydrochloride has been assayed in certain tablets by extracting the drug from the tablet matrix, by adjusting the pH to about 9.0 and then extracting immediately with chloroform. By measuring the IR absorption at 1720 cm^{-1} of the concentrated extract, the drug content can be quantitated. The method has also been applied to a syrup formulation assay.

7.11 Reineckate Salt

Methylphenidate hydrochloride has been determined gravimetrically by precipitating the reineckate salt of the free base by the addition of ammonium reineckate, $\text{NH}_4[\text{Cr}(\text{NH}_3)_2(\text{SCN})_4]$ to the drug in solution.

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NABILONE

Rex W. Souter

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1. Introduction

Nabilone, a totally synthetic 9-ketocannabinoid, is currently being evaluated to control nausea and vomiting in cancer chemotherapy patients (1-6) and as an ocular pressure-reducing agent in glaucoma patients (7,8). Such antiemetic (9) effects and ocular (10,11) effects have been reported for marihuana and the isolated natural product Δ^9 -tetrahydrocannabinol (Δ^9 -THC). Nabilone arose from an endeavor to discover drugs which would possess beneficial effects on the central nervous system while minimizing THC's disturbing side effects (12-14), especially tachycardia and dysphoria.

2. Description

2.1 Nomenclature

2.1.1 Chemical Name

(+)-trans-3-(1,1-Dimethylheptyl)-6,6a,7,8,10,10a-hexahydro-1-hydroxy-6,6-dimethyl-9H-dibenzo[b,d]pyran-9-one

2.1.2 Non-proprietary Name

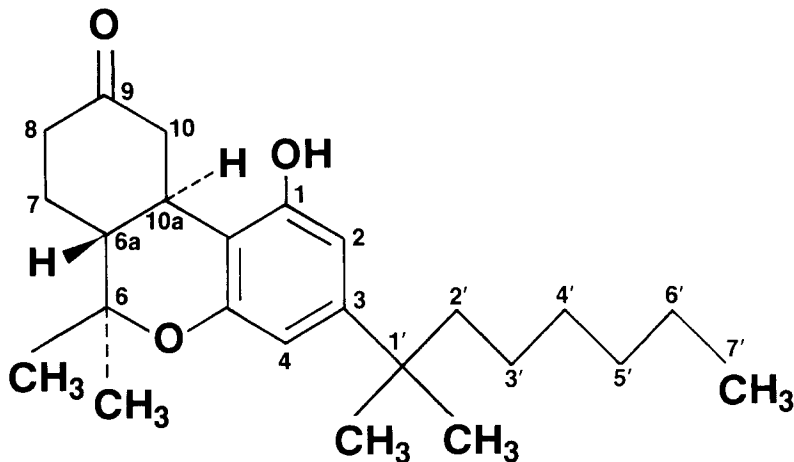
Nabilone

2.2 Formula

2.2.1 Empirical

$C_{24}H_{36}O_3$

2.2.2 Structural



2.3 Molecular Weight

372.5

2.4 Elemental Composition

<u>Element</u>	<u>% Theory</u>
C	77.38
H	9.74
O	12.88

2.5 Appearance and Odor

Nabilone is a white, crystalline solid having no particular odor.

2.6 Isomerism

2.61 Optical Isomers

Nabilone exists as a 1:1 mixture of 6aR, 10aR and 6aS, 10aS optical isomers and is therefore optically inactive (Cahn-Ingold-Prelog nomenclature).

2.62 Geometric Isomerism

The cis- isomer of nabilone is known to exist. This stereochemistry is defined by the position of the 6a hydrogen.

3. Physicochemical Properties

3.1 Spectra

3.11 Ultraviolet Spectrum

The ultraviolet spectrum (figure 1) in methyl alcohol from 240-360 nm exhibits maxima at 275 nm and 282 nm with molar absorptivities of 1284 and 1315 (and E 1%, 1 cm values both about 35) respectively. At 208 nm and 228 nm in methyl alcohol maxima also exists with molar absorptivities of approximately 10000 and 45800 (and E 1%, 1 cm values of about 269 and 1230) respectively. Figure 1 was plotted from data acquired with a GCA McPherson UV/Visible spectrophotometer.

3.12 Infrared Spectrum

The infrared spectrum of nabilone in a potassium bromide pellet (figure 2) was plotted from data taken from a Perkin-Elmer 580B instrument and the major band assignments are listed below.

<u>Infrared Absorption, cm⁻¹</u>	<u>Assignment</u>
3280, broad	-OH, hydrogen-bonded
2953	CH asym. in CH ₃
2925	CH asym. in CH ₂
2870, shoulder	CH sym. in CH ₃
2859	CH sym. in CH
1696	carbonyl stretch
1619 and 1574	phenyl C=C
1413	probably ring vibration

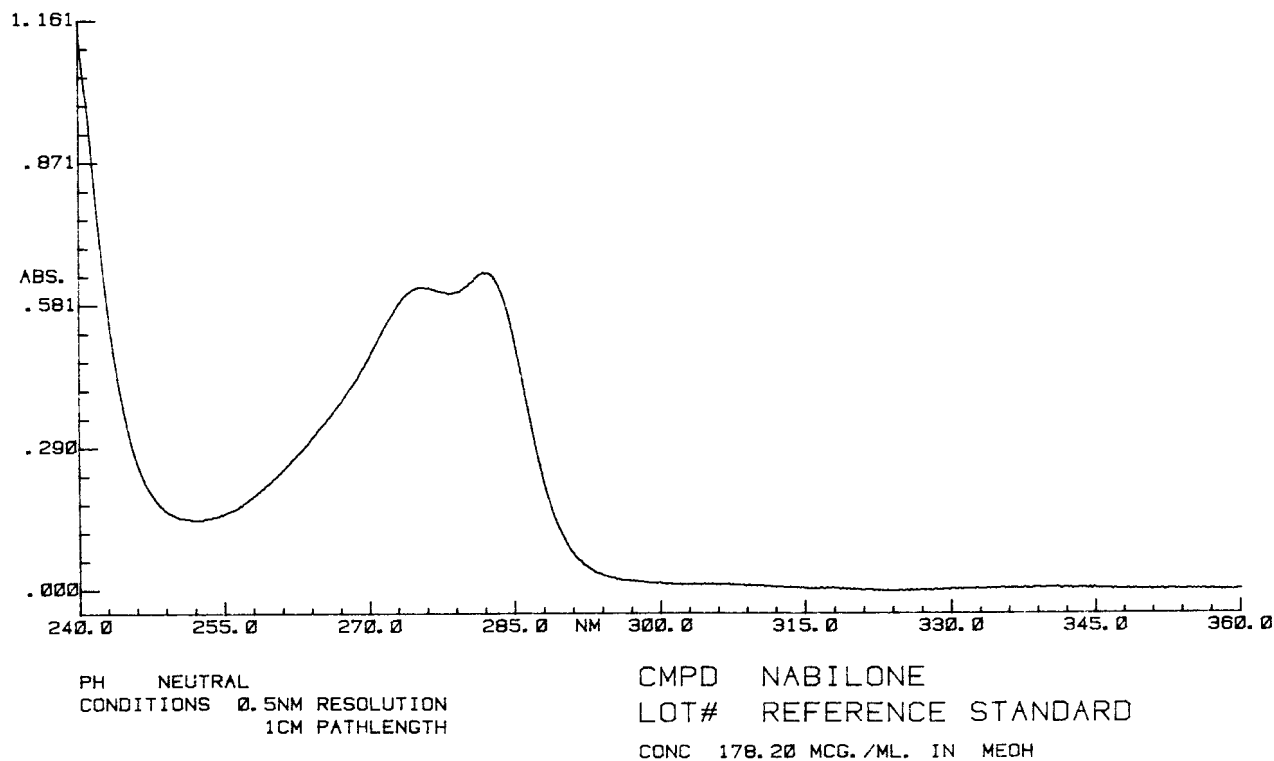


Figure 1. UV spectrum of nabilone

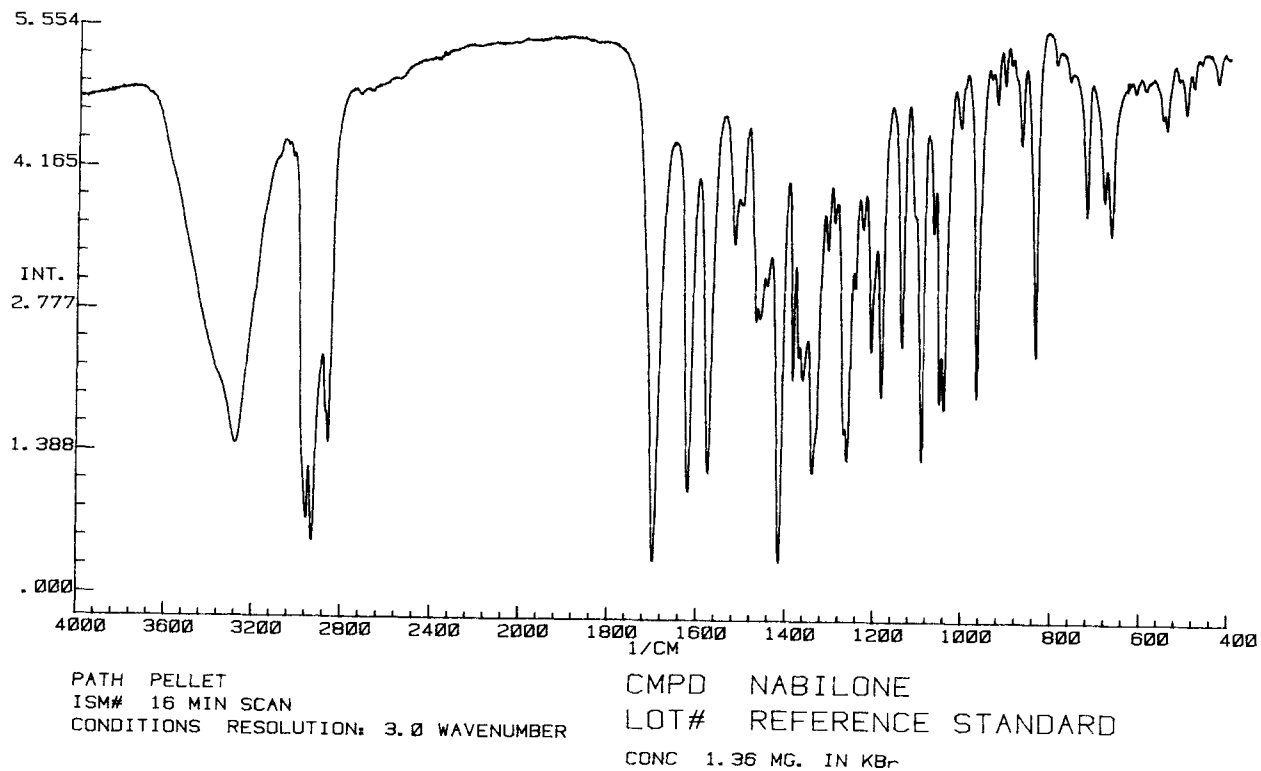


Figure 2. IR spectrum of nabilone

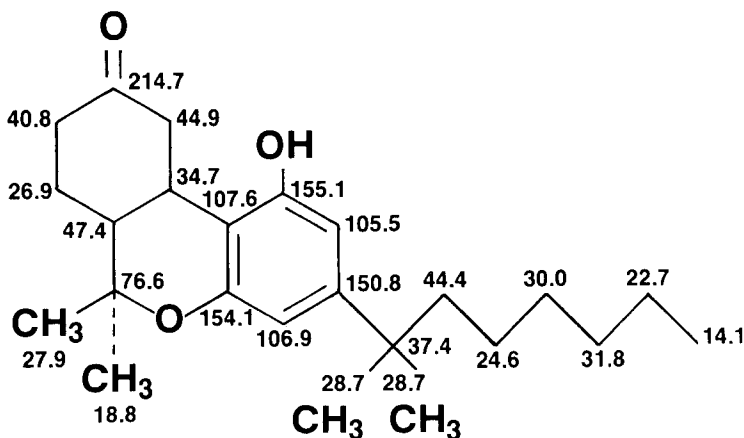
<u>Infrared Absorption, cm^{-1}</u>	<u>Assignment</u>
1385, 1371	CH deformation in $-\text{C}(\text{CH}_3)_2$
1363, 1359	ring and side chain
1340	includes $-\dot{\text{C}}\text{H}$ deformation
1260	C-O-C aromatic ether
1135	$-\dot{\text{C}}-\text{O}$ in polysubstituted phenyl
1038	$-\dot{\text{C}}-$ aliphatic ether
866	isolated CH, aromatic out-of plane deformation

3.13 Proton Magnetic Resonance Spectrum

The 60 MHz proton NMR spectrum of nabilone in deuteriochloroform (plotted with data from a Varian T60A spectrometer) is given in figure 3. With reference to the structure in 2.22 assignments of the resonances are also given.

<u>Description of Resonance</u>	<u>Assignment</u>
singlet, 1H, 7.75 ppm	-OH
broad singlet, 2H, 6.43 ppm	H (at 2 and 4)
doublet/doublet, 1H, 4.20 ppm	10 equatorial
triplet/doublet/doublet, 1H, 2.91 ppm	10 a
singlet, 3H, 1.50 ppm	β - CH_3 (6)
singlet, 6H, 1.23 ppm	CH_3 (1')
singlet, 3H, 1.15 ppm	α - CH_3 (6)
triplet, 3H, 0.85 ppm	7'

3.14 ¹³Carbon Magnetic Resonance Spectrum



The natural abundance ^{13}C NMR chemical shifts for nabilone are shown on the preceding page. All chemical shifts are in parts per million downfield from TMS in deuteriochloroform. The spectrum was recorded on a JEOL PFT-100 NMR spectrometer.

3.15 Mass Spectrum

Figure 4 is a plotted low resolution mass spectrum of nabilone from a Hitachi-Perkin Elmer RMU-6 instrument equipped with a System Industries System 150 data system. Fragmentation of the molecular ion of nabilone (m/e 372) along the alkyl side chain, with hydrogen rearrangement yields the homologous series of peaks m/e 330, 316, 302 and 288. M/e 288 arises by fission β to the phenyl ring, a process which may often lead to intense peaks in the spectra of alkyl benzenes. The origin of the above peaks can also be fragmentations of the alicyclic ring or combinations of alicyclic ring and side chain fragmentations. In cases where the keto group is eliminated, the possibilities can be distinguished by accurate mass measurement. M/e 177 has the composition $\text{C}_{11}\text{H}_{13}\text{O}_2$.

3.2 Melting Range

158-160°C

3.3 Crystallinity

3.31 Crystalline Habit

Nabilone may occur in at least four distinct polymorphic forms depending upon the solvent and crystallization conditions (15). The table below summarizes these forms which have been characterized by differential thermal analysis (DTA) and X-ray diffraction powder patterns.

<u>Polymorph</u>	<u>Crystallization Solvent</u>	<u>Endothermic DTA Transition Temperature</u>
A	hexane	162°C
B	ethanol-water ^(a)	155, 162°C
C	ethanol-water ^(b)	132, 155, 162°C
D	chloroform	120, 140, 162°C

(a) Crystallization allowed to occur from warm ethanol-water solution.

(b) Crystallization forced by the addition of ethanol solution to water.

3.32 X-ray Powder Diffraction

The data below describe the pattern for the most thermodynamically stable form (A) of nabilone where d is equal to the interplanar spacing measured in Angstroms (\AA) and I/I_1 are intensities of the x-ray maxima based on a value of 100 for the strongest line.

Cu-Ni Radiation, λ 1.5405 Å

<u>d</u>	<u>I/I₁</u>	<u>d</u>	<u>I/I₁</u>
9.27	100	3.56	30
8.00	50	3.43	05
6.78	30	3.28	50
6.63	30	3.12	02
6.21	40	3.07	02
5.85	10	2.92	02
5.58	05	2.72	02
5.17	70	2.62	02
4.88	90	2.44	10
4.73	15	2.39	05
4.58	20	2.33	10
4.29	30	2.24	02
4.06	40	2.18	05
3.89	60	2.04	02
3.73	40	1.89	05

3.4 Thermal Analysis

3.41 Differential Thermal Analysis (DTA)

A differential thermogram of nabilone at a heating rate of 5°C/min. in a nitrogen atmosphere (40cc/min) shows an exotherm at approximately 165°C indicating a melt.

3.42 Thermogravimetric Analysis (TGA)

A thermogram run simultaneously with the above DTA shows a weight loss beginning at 150°C resulting in a 0.6% loss at 180°C (residual solvent).

3.5 Dissociation Constant

The pka of nabilone in 66% dimethylformamide/34% water is 13.5.

3.6 Solubility Profile

The sample is sonicated for one minute at ambient temperature.

<u>Solvent</u>	<u>mg/ml</u>
Water	< 0.5
pH 1.2 (USP XIX)	< 0.5
pH 4.5 (USP XIX)	< 0.5
pH 7.0 (USP XIX)	< 0.5
methyl alcohol	>5.0
n-octyl alcohol	< 0.5
diethyl ether	>5.0
ethyl acetate	>10
chloroform	>10
benzene	>10
cyclohexane	> 1.0

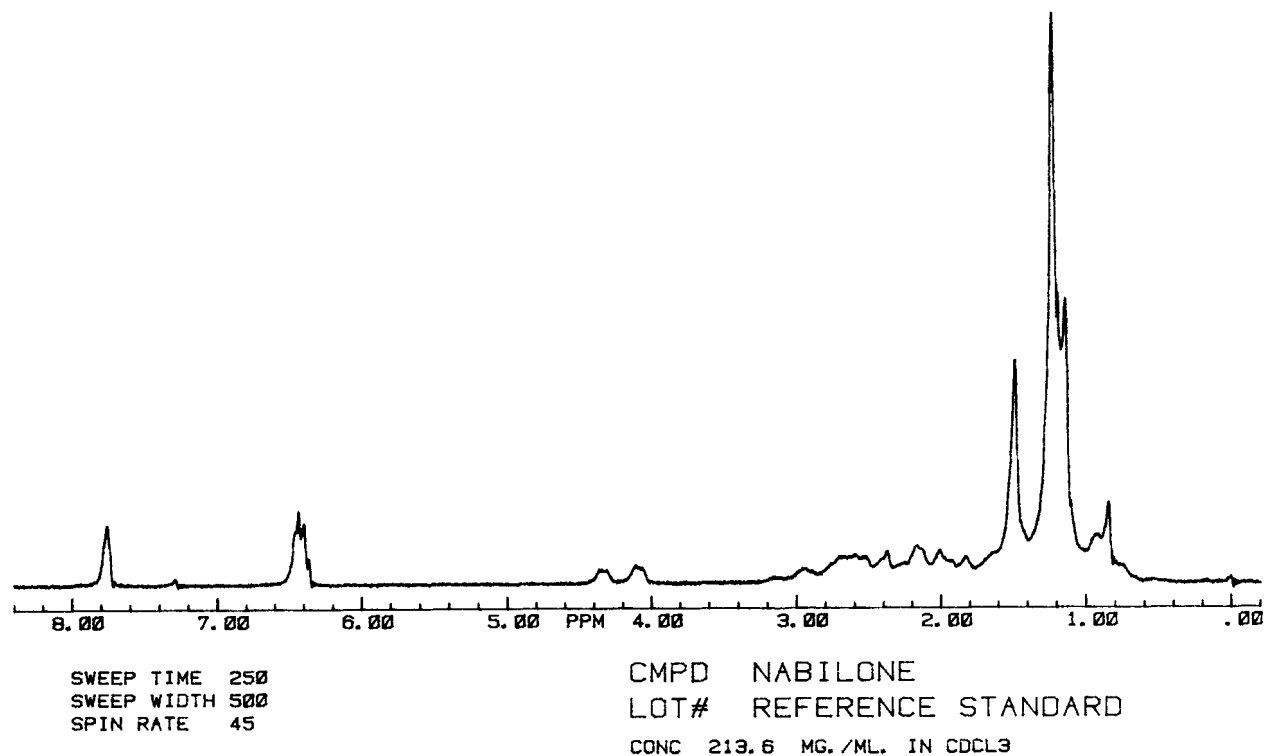


Figure 3. Proton nmr spectrum of nabilone

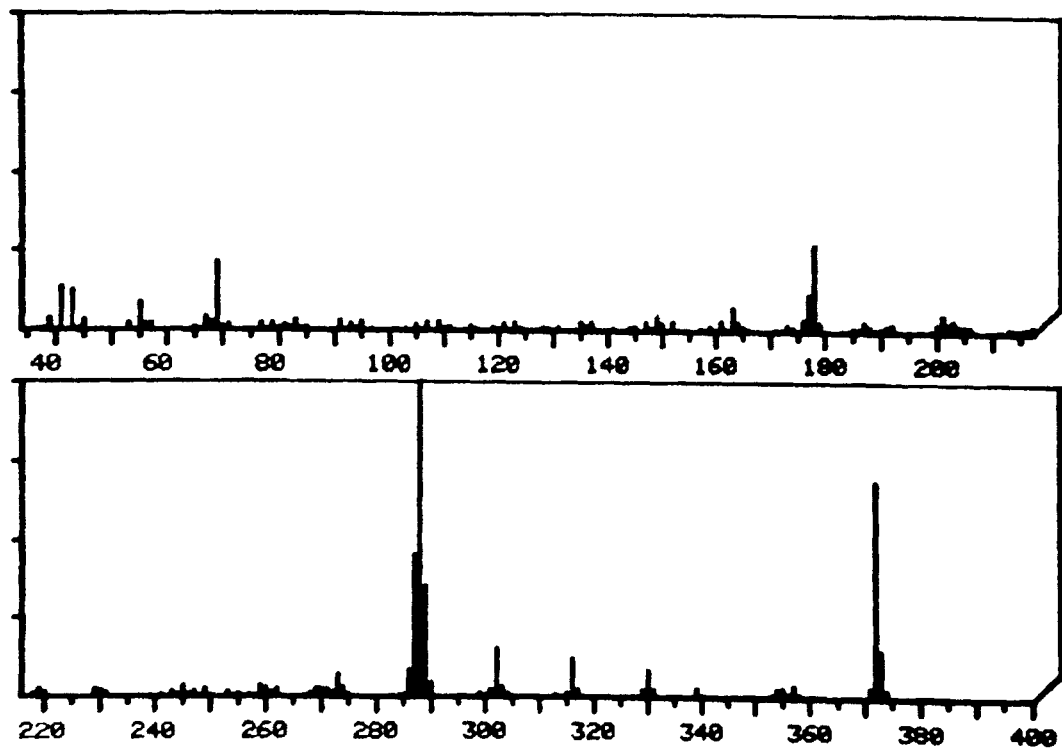
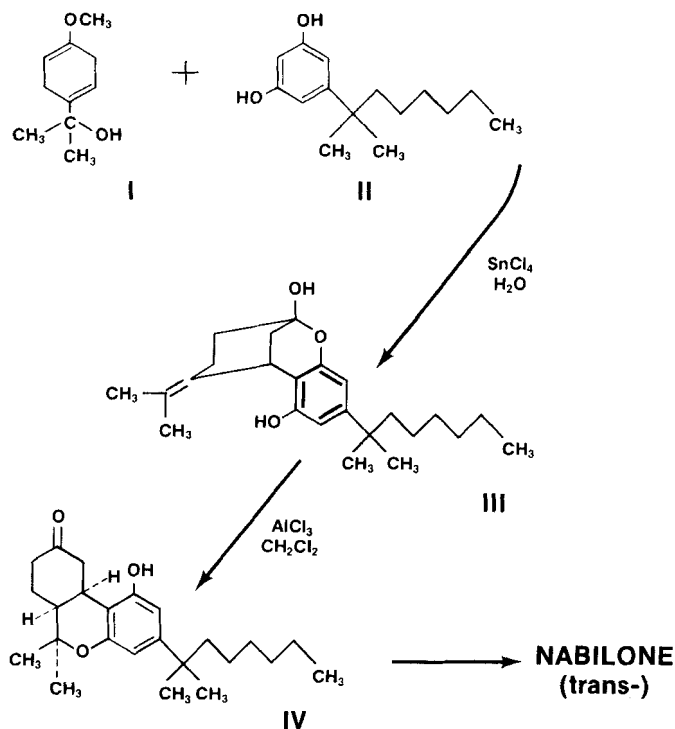


Figure 4. Low-resolution mass spectrum of nabilone

4. Synthesis

The resorcinol II may be reacted with diene I in the presence of water and stannic chloride. I may be prepared from p-methoxyacetophenone by Grignard addition to provide 2-(4-methoxyphenyl)-2-propanol, followed by Birch reduction. The reaction probably proceeds through a ketal which is hydrolyzed to the hemiketal III which subsequently rearranges to the cis-ketone IV. Ketone IV may finally be isomerized to nabilone by conversion with AlCl_3 in dichloromethane at 0°C (16), as shown below.

5. Stability5.1 Accelerated Degradation

Nabilone is stable to refluxing 0.1N acid and 0.1N base, as well as to heating in air at 110°C for one week. However, irradiation for just over two days in ethyl alcohol with a 200 W high pressure Vycor-filtered mercury arc yields primarily the cis and trans diols formed by reduction at the 9-keto position. Another observed product is the hemiketal (III in the synthetic diagram).

5.2 Long-Term Stability

Samples of nabilone stored up to four years under varying conditions of heat and humidity show essentially no change in potency.

6. Metabolism, Pharmacokinetics and Microbiological Transformations

Nabilone has been shown to have a plasma half-life of about two hours in man after intravenous and oral administration (17). Circulating metabolites included the isomeric carbinols formed by reduction at the 9-keto position.

Investigation of the pharmacokinetics of nabilone and its carbinol metabolites in the dog following the administration of small doses required development of a quantitative selected ion monitoring (SIM) gas chromatography mass spectrometry method (18). In the dog, nabilone was rapidly converted to a mixture of isomeric carbinols whose ratio in plasma was constant. The apparent half-life of nabilone in dogs was 1-2 hr. while that of the metabolite carbinols was of the order of 20 hr. following a single oral or I.V. dose. The SIM methods for nabilone and its metabolites have a lower sensitivity limit of about 2 pmol ml^{-1} with a coefficient of variation of less than 4% (18).

A screening program was used to find microorganisms capable of modifying nabilone (19) and nearly one-half of the tested microorganisms were found effective (19,20). Products from three cultures were characterized spectroscopically to determine their chemical structures (19).

7. Methods of Analysis

7.1 Raw Material

7.11 Gas Chromatography

Nabilone may be assayed using a glass column packed with OV-17 stationary phase on AW DMCS-treated chromosorb G. The sample is dissolved in a chloroform solution of the internal standard, methyl testosterone. Samples are compared to reference standards prepared in the same manner. The method exhibits a precision of about 0.7% (R.S.D.).

7.12 Thin Layer Chromatography (TLC)

Using a silica gel 60 F254 thin layer plate in a paper-lined chamber containing benzene/ethyl acetate 75:25, nabilone has a R_f of about 0.55. It may be visualized by exposing the plate to 254 nm UV light (so that the spot quenches the fluorescent indicator in the plate) or by spraying the dry plate with fast blue B spray reagent (21).

7.2 Biological Samples

Methods for assay of nabilone and its metabolites have been described (17,18).

7.3 Pharmaceutical Formulations

Following some steps to extract it from its capsule excipients, nabilone may be assayed by a gas chromatographic technique similar to that used for the raw material. Simple TLC identification of nabilone in capsules requires its extraction from excipients followed by TLC on a silica gel 60 F254 plate in a paper-lined chamber containing toluene/ethyl acetate 80:20. Nabilone has an R_f of approximately 0.37 in this system.

8. Acknowledgements

The assistance of Mr. Michael Gleissner in obtaining the ^{13}C nmr data is appreciated as are the efforts of Dr. A. D. Kossoy in performing and interpreting the accelerated degradation profile of nabilone. The author thanks Dr. L. G. Tensmeyer, Dr. D. E. Dorman, Mr. J. L. Occolowitz and Mr. C. D. Underbrink for their assistance in the acquisition and interpretation of the other spectral data. The constructive criticism of Dr. R. A. Archer in proofreading this manuscript is sincerely appreciated.

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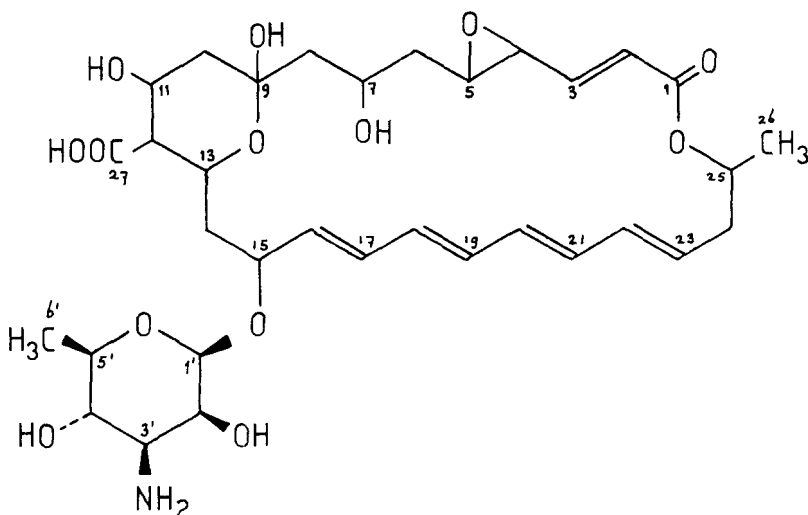
NATAMYCIN

Harry Brik

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1. Description1.1. Name, Formula, Molecular Weight

<u>Generic name</u>	natamycin
<u>Synonym</u>	pimaricin
<u>Trade names</u>	Natafucin; Pimafucin; Delvucid
<u>Chemical name</u>	22-(3-amino-3,6-dideoxy-β-D-mannopyranosyl)oxy-1,3,26-trihydroxy-12-methyl-10-oxo-6,11,28-trioxatri-cyclo[22.3.1.0 ^{5,7}] octacos-8,14,16,18,20-pentaene-25-carboxylic acid.

Structural formula*

<u>Empirical formula</u>	C ₃₃ H ₄₇ NO ₁₃
<u>Molecular weight</u>	665,75
<u>Code designation</u>	CL 12 625; Antibiotic A-5283
<u>CAS registry number</u>	7681-93-8
<u>Wiswesser line notation</u>	T F3-24-6 A AO GO KVO IU OU QU SU UUTJ BQ DQ M1 C&VQ D&Q WO-BT60TJ CQ DZ EQ F1

*In contradiction to the IUPAC chemical name the C-atoms in the structural formula are numbered in the classical way to facilitate comparison with literature data which refer to parts of the molecule.

1.2. Chemical Structure and Configuration

Natamycin belongs to the large group of polyene antifungal antibiotics. Characteristic for this group is a macrocyclic lactone-ring with a number of conjugated carbon-carbon double bonds. The chemical structure of natamycin was proposed at first by Patrick et al in 1958 (1,2). Six years later an extensive reinvestigation was presented by Ceder (3). A minor revision (absence of an OH-group at C₈) of Ceder's proposed structure was made by Golding et al in 1966 (4). This last structure (Section 1.1) was confirmed by Haegeler et al (5) using mass spectrometry of the per-trimethylsilyl derivative and by Ceder et al (6) using ¹³C-NMR spectroscopy of i.e. the N-acetyl derivative.

The hemiketal-structure of the C₉-C₁₃ part of the lactone-ring was confirmed by optical rotation dispersion (7,8) and by proton-NMR spectroscopy (6).

The absolute configuration at C₂₅ was established as R by isolation of an optically active derivative of the C₂₄-C₂₆ chain (9,10).

The total configuration of the major parts of the molecule (Figure 1) was elucidated by Ceder et al (6) using proton- and ¹³C-NMR spectroscopy. From the same experiments the diastereometrical purity of natamycin could be deduced as well.

On the basis of ultraviolet data the tetraene system has been shown to be all-trans (11).

Structurally, natamycin is closely related (Figure 2) to the tetraenes lucensomycin (12,13), arenomycin B (14) tetramycin (15) and the tetrins A and B (16,17). Contrary to most other polyenes, natamycin contains little or no congeners.

1.3. Nomenclature

The natamycin-producing *Streptomyces* strain was found in a soil sample from the neighbourhood of Pietermaritzburg, a town in the province of Natal, South Africa. Therefore the strain in question was called *S. natalensis*.

The name natamycin is commemorative of this strain, the old name pimarinin (still used sometimes but not accepted by the WHO since antibiotics which are produced by a *Streptomyces* strain should have the suffix "mycin") is called after Pietermaritzburg.

The synonym tennecetin (Section 4.1) is no longer used.

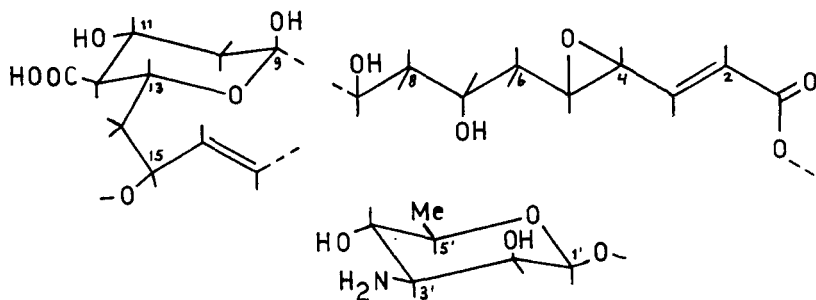


Figure 1. Configuration of the major parts of natamycin according to Ceder (6).
 mycosamine : absolute configuration
 C₁-C₁₇ fragments: relative configuration

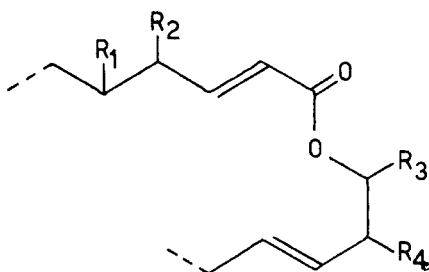


Figure 2. Structural relation of natamycin to other tetraenes which differ only in R₁ - R₄

tetraene	R ₁	R ₂	R ₃	R ₄
natamycin	-O-		Me	H
lucensomycin	-O-		n-Bu	H
arenomycin B	OH	H	n-Bu	H
tetramycin	OH	H	Me	Et
tetrin A	OH	H	Me	Me
tetrin B	OH	OH	Me	Me

1.4. Appearance

Natamycin is a white to cream-coloured, almost tasteless and almost odourless, crystalline powder.

1.5. Standards and Regulatory Status

The microbiological activity of natamycin is expressed in μg per mg . The FDA master standard has an assigned potency of 922,0 μg of anhydrous natamycin per milligram (18).

This standard is equal to Gist-Brocades natamycin trihydrate reference standard lot 711-EN-78-1.

The USP reference standard (lot F, catalog number 4575) is equal to Gist-Brocades natamycin trihydrate reference standard lot 705-EN-84-1.

Both standards were purified by repeated crystallization of a selected lot of natamycin.

1.6. Antimycotic Properties

Natamycin is effective against a broad variety of fungi, yeasts, some protozoa and some algae. It has no antibacterial activity. Natamycin is used topically against fungal infections of the skin and the mucous membranes in the form of several dosage forms (suspensions, creams, ointments and vaginal tablets) alone or in combination with neomycin and hydrocortisone or other steroids. A summary of the therapeutic use of natamycin is given by Raab (19).

Natamycin is also used as a food additive, mainly as an antimycotic on cheese, meat products and in wines and fruit juices. The antimycotic action on foods is twofold, it prevents economic losses as well as the formation of mycotoxins (20,21). In wine it can replace sorbic acid and other antifungal agents and it allows a reduction of the amount of sulfur dioxide used (22). Compared with classical antifungal agents natamycin is active in very low concentrations, on cheese for instance it is 400 times more active than potassium sorbate (23).

A review on the use of natamycin in foods is given by Morris (24).

The application of natamycin as a food additive besides its use as a drug is possible due to a number of favourable properties: it has a very low oral toxicity, absorption through the intestine has not been demonstrated, sensitizing properties have not been found and (cross)resistance has never been encountered (see Section 6.1).

2. Chemical Properties

The amphoteric character of natamycin is responsible for its low solubility in most solvents. The solubility in water or lower alcohols is increased at low and high pH. Instable, crystalline salts, like the potassium salt and the sulphate are known (25).

Improved solubility in water without sacrificing any stability or microbiological activity can be achieved by complex formation with boric acid (26) or a modified polysaccharide (27). This is also achieved by chemical modification, for instance by formation of alkyl esters (28,29,30), amides (31) and N-glycosyl derivatives (32).

Natamycin forms a 1:1 ion-pair with kation tensides (33) which, unlike the parent compound, is soluble in less-polar solvents (Section 6.4).

The complex formation of natamycin with sterols like cholesterol and especially ergosterol (34) is the basis of both its fungicidal action and the antagonistic properties of the above sterols (35).

The tetraene chromophore gives the molecule a highly unsaturated character. It reacts readily with bromine and compounds containing active oxygen such as permanganate, persulphate and peroxides. On the other hand natamycin contains weakly active oxygen in the form of an epoxy-group. The latter liberates iodine when natamycin is treated with a hot solution of iodide in glacial acetic acid (3).

The aminosugar mycosamine is liberated by acid hydrolysis, the lactone is saponified by alkaline hydrolysis. See also Section 5.

3. Physical Properties

3.1. Crystal Properties

3.1.1. Optical Crystallographic Data

Natamycin crystallizes from aqueous lower alcohols in the form of the trihydrate as thin rectangular plates (Figure 3) showing the following optical constants (33):

refractive indices	: 1,540 and 1,698
extinction	: parallel
optic sign	: positive
axial angle 2V	: < 15°

No polymorphism has been reported for the trihydrate.

Natamycin methanol solvate crystallizes from a saturated methanolic solution of the trihydrate as thin, strongly birefringent needles with parallel extinction and positive elongation.

Upon contact with water the solvate rapidly converts to the trihydrate. The latter shows the same X-ray diffraction pattern as the trihydrate crystallized from aqueous solvents (36). The above optical constants are also identical for both forms. However, the trihydrate obtained from the solvate as indicated above, has a habit which is somewhat different from the usual trihydrate. Unlike the latter (merely rectangular plates) the recrystallization product consists predominantly of prisms terminated at one end by a pyramid, with a negative sign of elongation, showing transitions to rectangular plates.



Figure 3. Natamycin crystals from aqueous propanol.
1 division = 10 μm

3.1.2. X-ray Crystallographic Data

Hempel et al. (37) determined the crystal structure of natamycin by X-ray analysis utilizing $\text{CuK}\alpha$ radiation. The following results were obtained:

crystal structure	: monoclinic
space group	: $P2_1$
cell dimensions	: $a = 0,768(1) \text{ nm}$ $b = 0,875(1) \text{ nm}$ $c = 2,690(2) \text{ nm}$
β angle	: $92,3(2)^\circ$
unit cell volume	: $1,805 \text{ nm}^3$
molecules per unit cell	: 2

3.2. Ultraviolet spectrum

The ultraviolet spectrum of natamycin (USP reference standard, lot F) in methanol with 0,1% acetic acid is shown in Figure 4. The acetic acid acts as a "wavelength stabiliser" as traces of alkali introduce a small red-shift of up to 2 nm. This principle is used in the spectrophotometric analysis of natamycin (Section 7.2).

The spectrum exhibits sharp maxima at 290, 303 and 318 nm, a shoulder at 280 nm and a broader maximum at 220 nm. Under the above conditions the following spectral constants were obtained (33):

Table 1
ultraviolet data

λ_{max} (nm)	$A_1^{1\%*}$ cm	mol. abs. coeff.
220	320	21 300
280	400	26 630
290	795	52 930
303	1250	83 220
318	1145	76 230

*calculated with reference to the dried substance

The absorption bands in the region between 280 and 320 nm are characteristic for an all-trans tetraene (11), the maximum at 220 nm is attributed to the en-one chromophore.

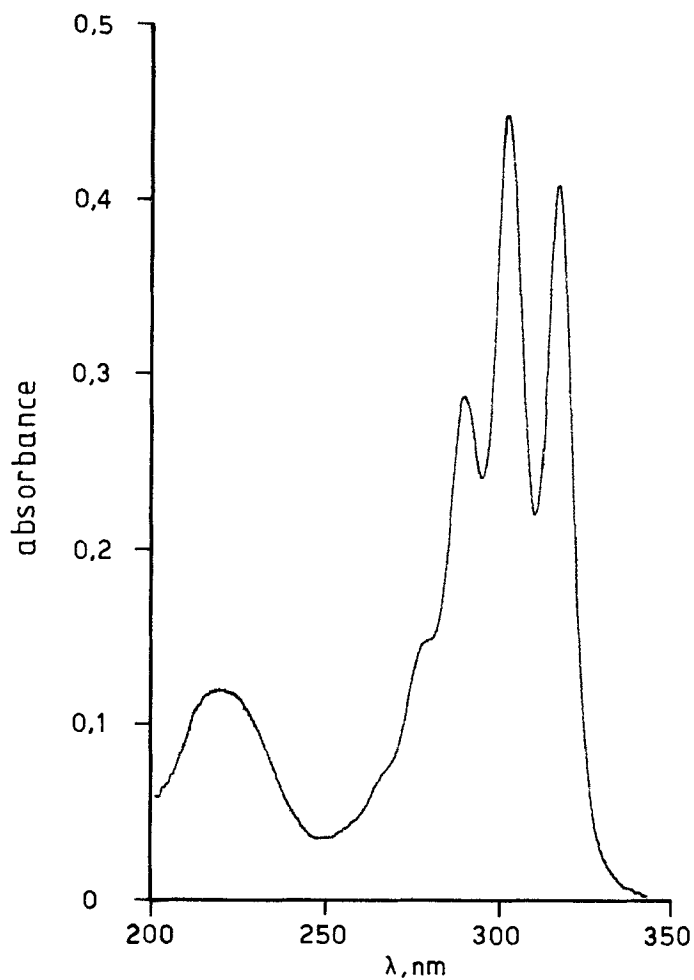


Figure 4. Ultraviolet spectrum of natamycin in methanol with 0,1% acetic acid at a concentration of 3,6 μg (calculated on the anhydrous basis) per ml.
Instrument used: Beckman Acta CIII

Several authors (25,38,39,40) report somewhat lower molar absorption coefficients for the tetraene chromophore.

The ultraviolet spectrum of natamycin is similar to the spectra of tetraene antibiotics which belong to the same sub-group having the same chromophores, especially its C₂₅ butyl-homologue lucensomycin (12), further the closely related arenomycin B (14), tetramycin (15), tetrin A (16) and tetrin B (17). The four latter tetraenes however lack an epoxy-group. It is possible that for this reason the en-one chromophore of these tetraenes absorbs at somewhat lower wavelengths (208-212 nm).

The absorption at 220 nm differentiates natamycin from the tetraenes nystatin and amphotericin A (see Section 7.1).

3.3. Infrared spectrum

The infrared spectrum of natamycin (USP reference standard lot F) in a potassium bromide pellet (41) is presented in Figure 5.

A spectrum of the same sample in liquid paraffin is essentially identical to the one presented.

An interpretation of the spectrum is given in Table 2.

Table 2
Infrared spectral assignments (42)

wavenumber (cm ⁻¹)	assignment
1005	CH def. in CH=CH
1060	C-O-C
1110	C-OH asymm. stretch
1270	C-O-C epoxy
1400	CH stretch
1570	CH=CH stretch; COO ⁻
1715	C=O lactone
2950	CH ₂ stretch
3020	=CH stretch
3270	NH ₃ ⁺
3500	OH
3600	OH hemiketal
2400 - 3600	OH - carboxyl; water

When preparing a KBr pellet it is important to evacuate for not more than 10 seconds to avoid distortion of the spectrum due to loss of water of crystallization (41).

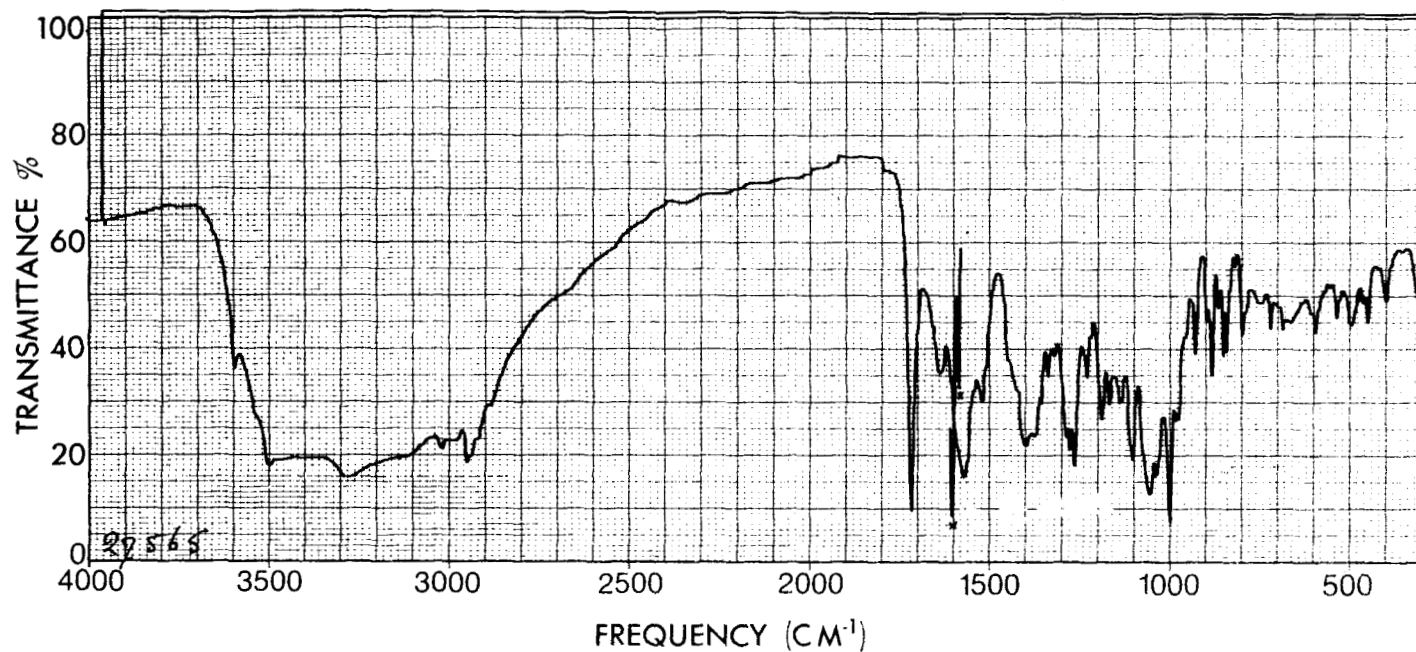


Figure 5. Infrared spectrum of natamycin trihydrate in a KBr pellet (x = polystyrene calibration points at 1600 and 1583 cm⁻¹)
Instrument used: Perkin-Elmer Model 521

3.4. Proton NMR Spectrum

DeBruyn et al. (43) have recorded a 300 MHz proton NMR spectrum of natamycin (Gist-Brocades reference standard 705-EN-71-1) in deuterio-trifluoroethanol containing some NaOD. The assignments are given in Table 3.

Table 3
Assignments of the peaks in the 300 MHz
proton NMR spectra of natamycin (43)

chemical shift, ppm	proton	chemical shift, ppm	proton
1,27	6B	3,22	5'
1,37	6', 26	3,92	2'
1,40	10B	4,25	11, 13
1,73	8B	4,36	15
1,83	8A, 14B	4,50	7
2,12	6A	4,57	1'
2,13	10A, 12	4,75	25
	14A	5,67	23
2,26	24B	5,93	16
2,40	24A	6,08	2
2,65	3'	\pm 6,25	17-22
2,97	5	6,47	3
3,21	4, 4'		

However, the resolution in this solvent is rather poor (see Figure 6) and better results can be obtained using the N-acetyl derivative in pyridine- d_5 . Ceder et al (6) published a 270 MHz spectrum and assigned most of the resonances and coupling constants. The spectrum could be completely assigned except for part of the tetraene moiety ($H_{18} - H_{20}$) using more sophisticated resolution enhancement techniques on a Bruker WM 250 (44). See Figure 7 and Table 4.

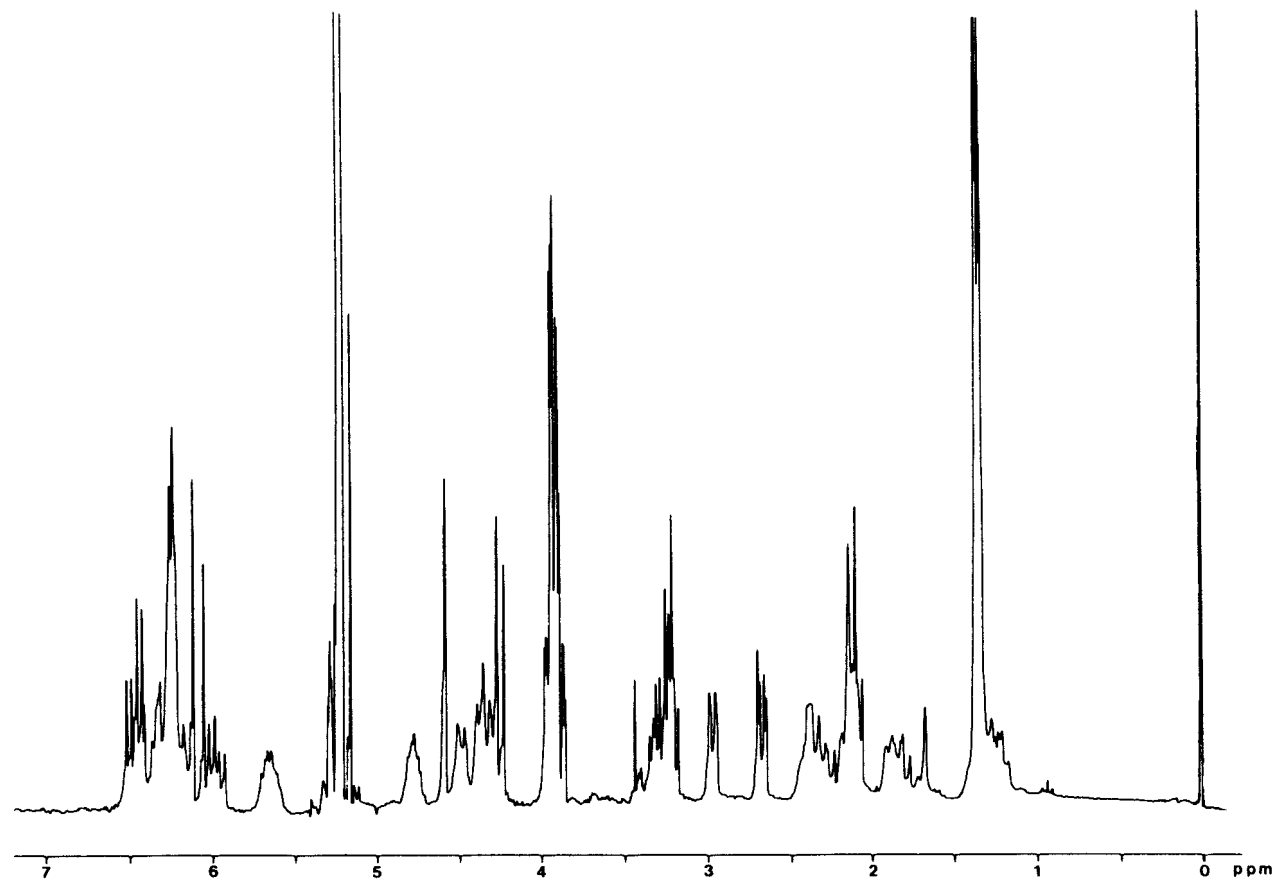


Figure 6. 250 MHz proton NMR spectrum of natamycin (USP reference standard) in deuterioethanol/NaOD (44)

Table 4

Assignments of the peaks and coupling constants in the 250 MHz proton-NMR spectrum of N-acetylratamycin (44)

chemical shift, ppm	proton	chemical shift, ppm	proton
1,256	26	4,523	2'
1,431	6A	4,583	3'
1,517	6'	4,658	7
1,814	8A	4,838	25
1,836	10A	4,958	15
1,956	8B	5,083	1'
2,036	acetyl	5,202	11
2,050	6B	5,266	13
2,107	14A	5,572	23
2,144	24A	6,173	22
2,222	24B	6,188	18-20
2,582	10B	6,200	21
2,802	14B	6,352	17
2,953	12	6,358	2
3,167	5	6,490	16
3,291	4	6,669	3
3,641	5'	8,751	NH
4,023	4'		

Coupling constants, Hz

$3J(1',2')$	< 0,3	$3J(6B,7)$	3,5	$3J(14A,15)$	2,2
$3J(2',3')$	3,3	$3J(7,8A)$	2,5	$3J(14B,15)$	4,4
$3J(3',4')$	9,9	$3J(7,8B)$	10,7	$3J(15,16)$	8,1
$3J(4',5')$	9,4	$2J(8A,8B)$	14,7	$3J(17,18)$	10,5
$3J(5',6')$	6,2	$2J(10A,10B)$	12,5	$3J(21,22)$	11
$3J(3',NH)$	8,3	$3J(10A,11)$	10,5	$3J(22,23)$	15,5
$3J(2,3)$	16,0	$3J(10B,11)$	4,8	$3J(23,24A)$	9,8
$3J(3,4)$	7,8	$3J(11,12)$	10,2	$3J(23,24B)$	6,4
$3J(4,5)$	2,2	$3J(12,13)$	10,8	$2J(24A,24B)$	13,5
$3J(5,6A)$	8,1	$3J(13,14A)$	8,5	$3J(24A,25)$	10,1
$3J(5,6B)$	< 1	$3J(13,14B)$	< 1	$3J(24B,25)$	3,2
$2J(6A,6B)$	15,0	$2J(14A,14B)$	15,2	$3J(25,26)$	6,4
$3J(6A,7)$	11,1				

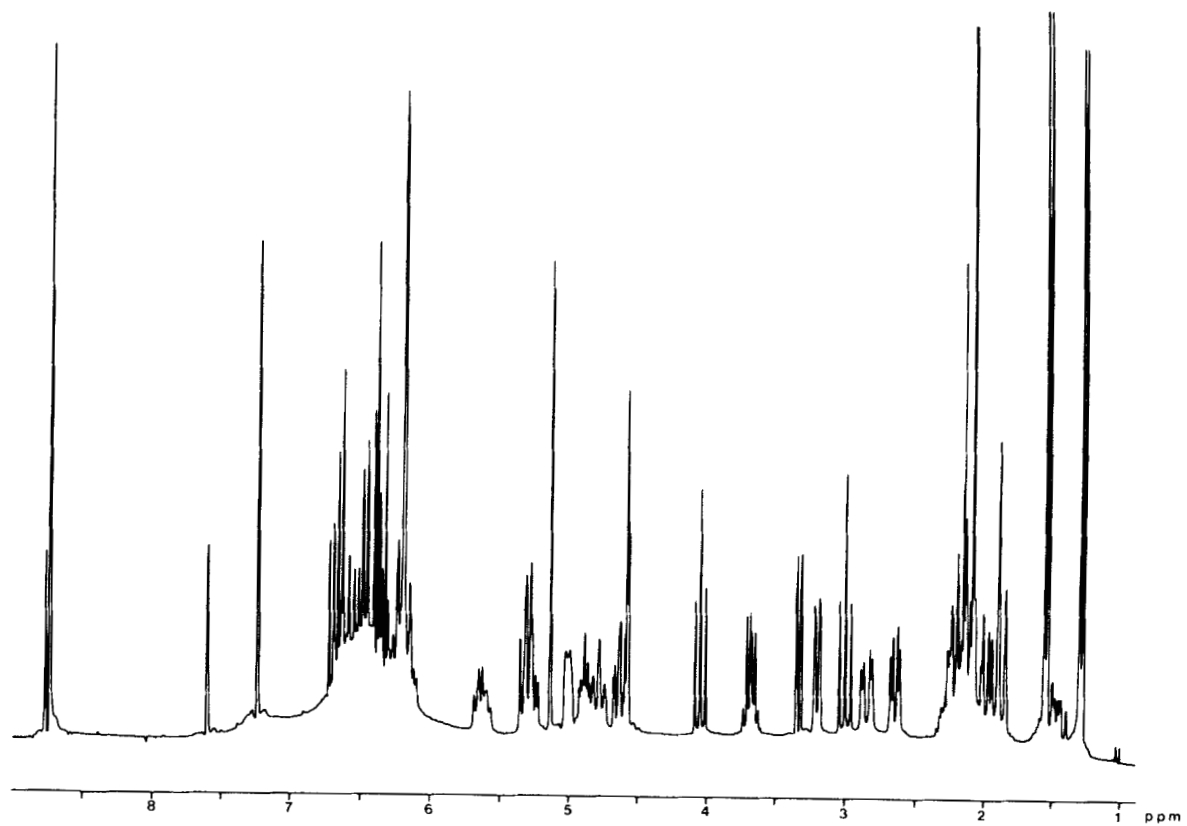


Figure 7. 250 MHz proton NMR spectrum of N-acetylbatamycin (44) in pentadeuteropyridine

3.5. ^{13}C -NMR Spectrum

Pandey et al. (45) obtained ^{13}C -NMR spectra of natamycin and N-acetylnatamycin in DMSO, but they assigned only four of the resonances, which confirmed the hemiketal structure. The ^{13}C -NMR spectrum of natamycin (62,89 MHz) in deuterio-trifluoroethanol/NaOD is shown in Figure 8 and the assignments are given in Table 5 (44). As in the proton NMR spectrum the resolution is poor and not all peaks are separated.

Table 5
Assignments of the peaks in the ^{13}C -NMR
spectrum of natamycin (44)

chemical shift, ppm	C-atom	chemical shift, ppm	C-atom
18,23	6'	74,76	4'*
20,62	26	75,39	5*
41,16	6,10,14	81,58	15*
44,54	24	99,73	9
47,84	8	100,83	1'
56,45	4	126,32	2
57,16	3'	130,70	16**
60,20	5	133,22	17**,18**,19**
61,14	12	134,35	20**
68,22	13*	134,59	21**
68,64	11*	135,12	22**
69,54	7*	137,45	23**
71,96	25*	145,92	3
73,08	2'*	168,82	1
		182,25	27

*,** assignments may be interchanged

Ceder et al. (6) assigned part of the ^{13}C -NMR spectrum of N-acetylnatamycin in pyridine- d_5 by comparison with the spectra of the dodecahydroderivative and the hydrogenation-hydrogenolysis product. Using selective decoupling techniques, all resonances of the ^{13}C -NMR spectrum of N-acetylnatamycin (62,89 MHz) could be assigned except for part of the tetraene moiety ($\text{C}_{18}\text{-C}_{22}$) (44). See Figure 9 and Table 6.

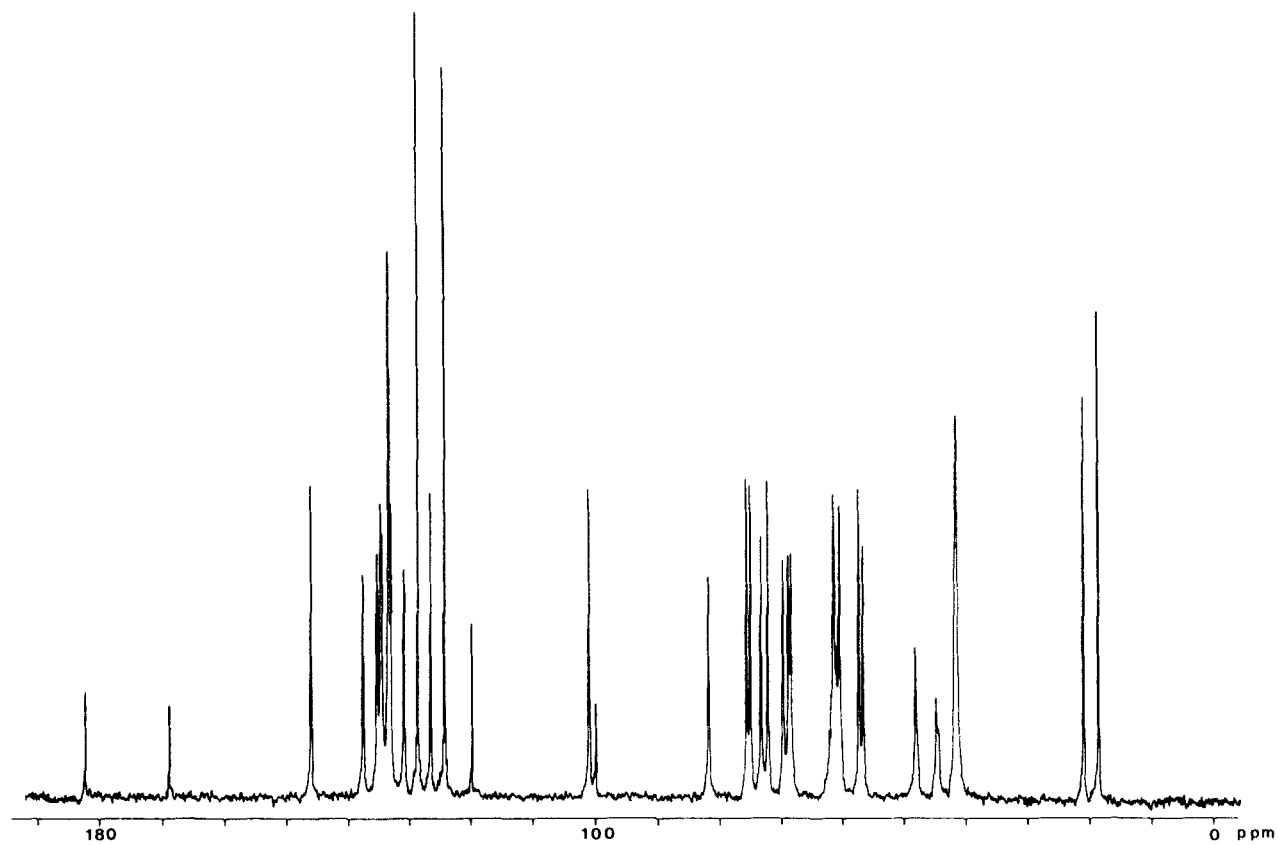


Figure 8. ^{13}C -NMR spectrum of natamycin (USP reference standard) in deuterioethanol/NaOD

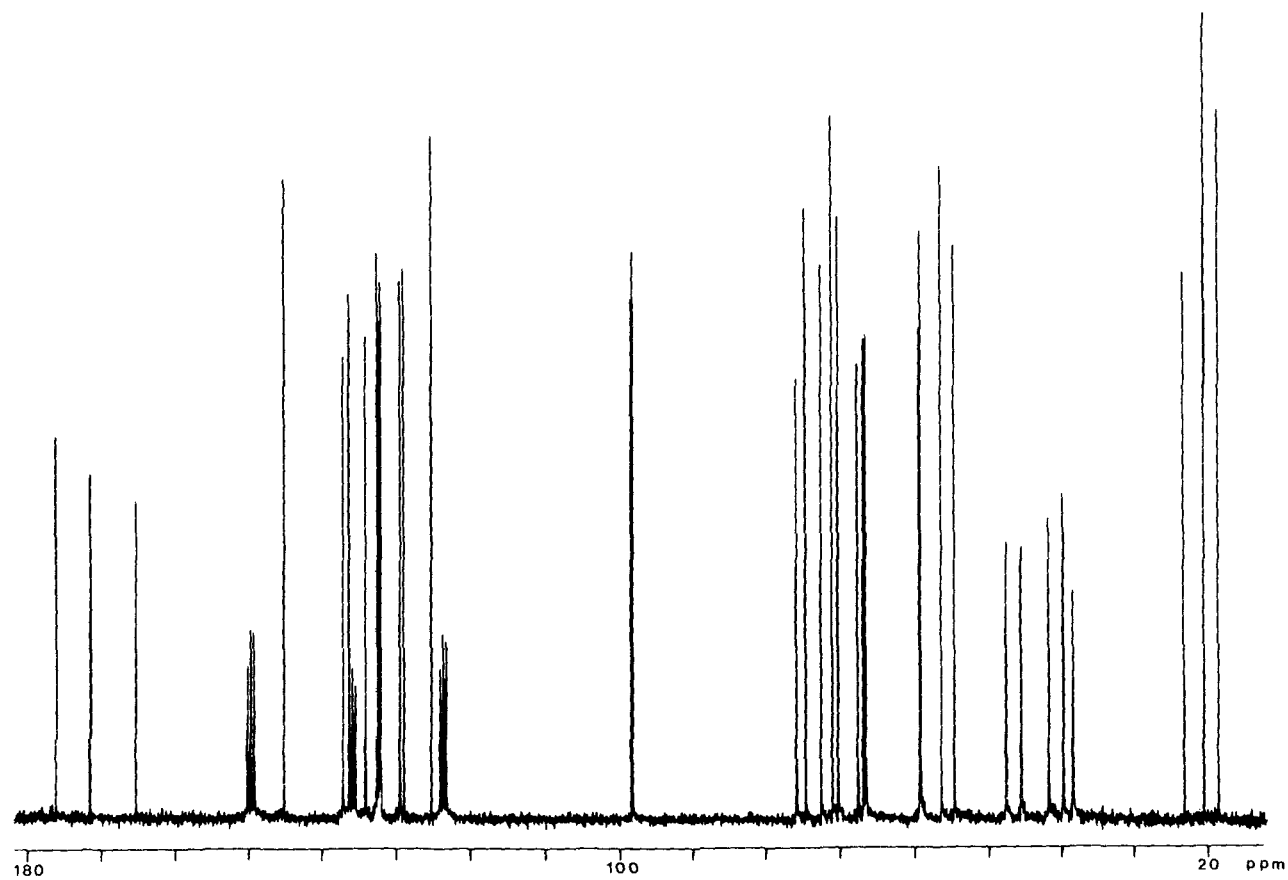


Figure 9. ^{13}C -NMR spectrum of N-acetylratamycin in pentadeuteropyridine (44)

Table 6
Assignments of the peaks in the ^{13}C -NMR
spectrum of N-acetylnatamycin (44)

chemical shift, ppm	C-atom	chemical shift, ppm	C-atom
17,55	6'	73,60	5'
19,45	26	74,77	15
22,17	8'	97,22	1'
37,39	14	97,46	9
38,77	10	124,24	2
40,76	6	127,95	23
44,58	24	128,47	17
46,65	8	131,10	18*
53,66	4	131,30	19*
55,42	3'	131,55	20*
58,16	5	133,06	16
58,34	12	135,29	21*
65,54	13	136,11	22*
65,82	11	144,15	3
66,57	7	164,36	1
69,21	25	170,27	7'
70,00	2'	175,25	27
71,47	4'		

* assignments may be interchanged

3.6. Mass Spectrum

Dornberger et al. (46) and Ceder et al. (6) obtained mass spectra of underivatized natamycin using electron impact and field desorption mass spectrometry. In this way a molecular ion is not obtained, the highest mass observed corresponds to $\text{M} - \text{mycosamine} - \text{CO}_2 - 2 \text{H}_2\text{O}$.

Falkowski et al. (47) obtained field desorption mass spectra of several natamycin derivatives.

By means of mass spectrometry of the pertrimethylsilyl derivative (5,48) the molecular ion at m/e 1169 of the fully silylated derivative was obtained. The mass spectrum presented in Figure 10 was run on a Varian MAT 311A by direct sample introduction at 180°C . The ionizing energy was 70 eV and the accelerating voltage 2 kV (48). The fragmentation pattern, as proposed by Haegele et al. (5) and corroborated by deuterated TMS - and acetyl - derivatives, is shown in Figure 11.

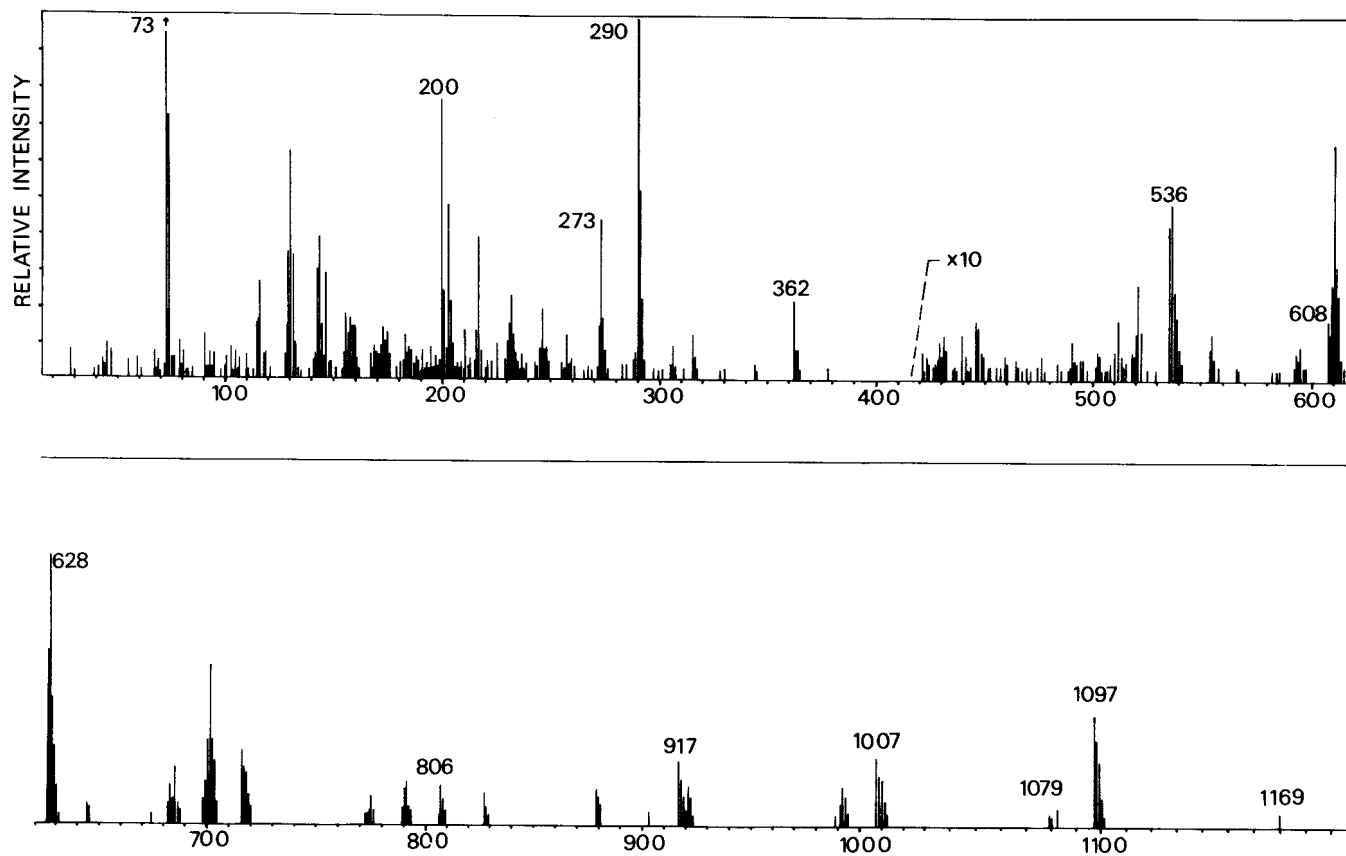


Figure 10. Mass spectrum of per-TMS natamycin (48)

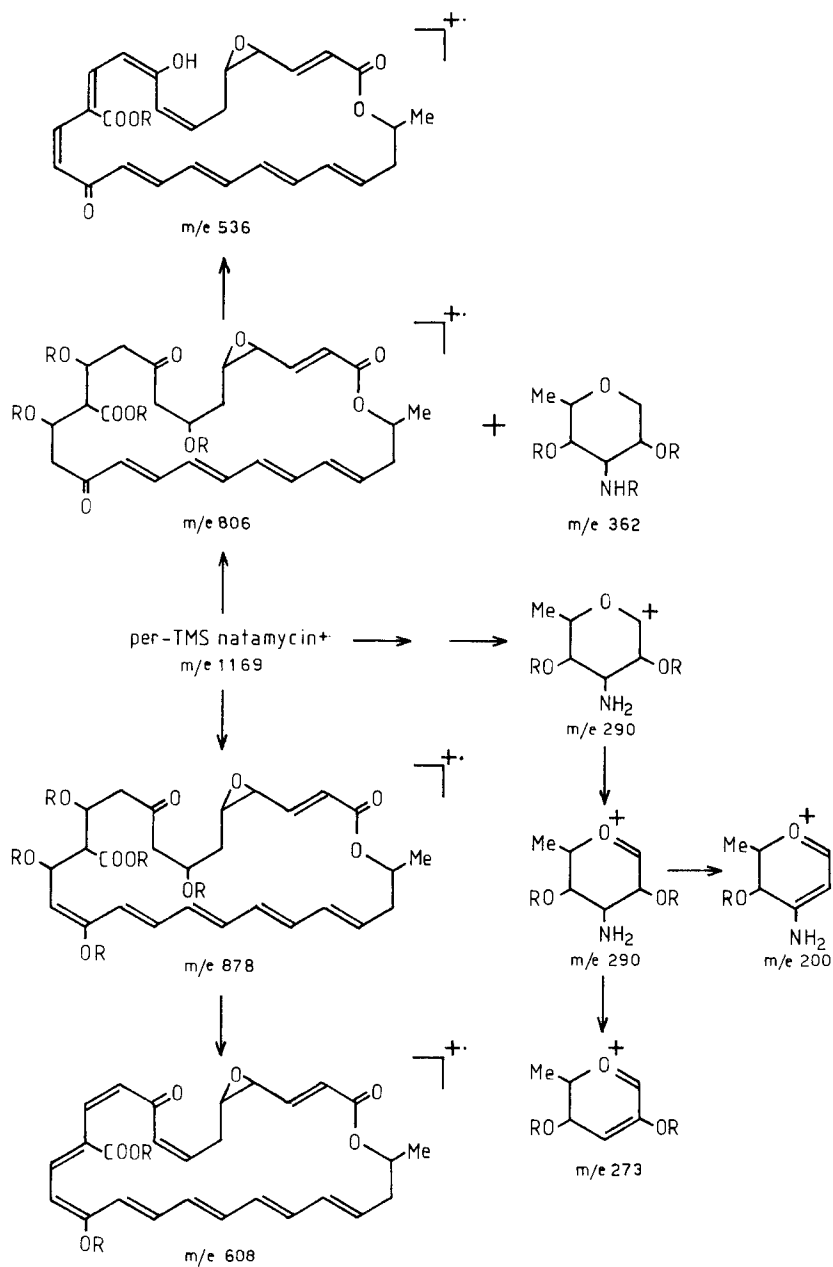


Figure 11. Fragmentation pattern of natamycin.
 R = trimethylsilyl (according to ref. 5)

3.7. Optical rotation

Specific rotation values from different sources are shown in table 7.

Table 7
Specific Rotation of natamycin

Solvent	conc.	temp.	$[\alpha]_D$	reference
dimethylsulphoxide	0,5%	27°C	+ 180°	39
dimethylformamide		25°C	+ 258°	25
acetic acid		25°C	+ 273°	19
acetic acid	1,0%	20°C	+ 278°*	49
pyridine	0,5%	20°C	+ 294°	49
methanol	0,08%	25°C	+ 250°	38

*mean value of 7 reference preparations, calculated with reference to the dried substance, equivalent to +257° for the trihydrate.

3.8. Optical Rotary Dispersion

The optical rotary dispersion (ORD) curve of natamycin (7) is reproduced in Figure 12.

From the shape of the ORD curve of natamycin and other polyenes Chong et al. (7) and Dornberger et al. (8) concluded that natamycin - in methanolic solution - exists as a cyclic hemiketal rather than the open hydroxy-ketone form.

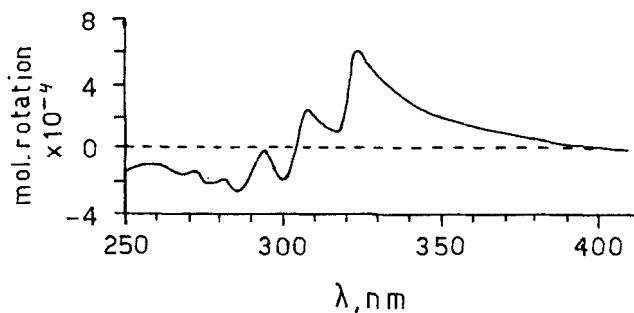


Figure 12. Optical rotary dispersion curve of a methanolic solution of natamycin (taken from ref. 7).

3.9. Ionization Constants

By titration of natamycin in 50% aqueous methoxyethanol, apparent pKa values of 8,35 and 4,6 were obtained (33), corresponding with an isoelectric point of 6,5 [also reported by Raab (50)]. The pH of a 1% aqueous suspension in water is accordingly normally between 5,0 and 7,5.

3.10. Thermal Behaviour

3.10.1. Melting Range

As most other amphoteric polyenes natamycin has no sharp melting point. It darkens at about 200°C and "melts" under vigorous decomposition at 280 - 300°C.

Natamycin, when heated on a Leitz Model 350 microscope hot stage at a rate of 5°C/minute and viewed in polarized light, showed a transition at 195 - 200°C to an isotropic modification or degradation product (33).

3.10.2. Differential Thermal Analysis (DTA)

A DTA curve of natamycin trihydrate was run on a Mettler TA 2000 differential thermoanalyzer between roomtemperature and 230°C (51). The operating conditions were as follows:

sample weight	2,32 mg
sample atmosphere	nitrogen
heating rate	5°C/min.
range	100 μ V fsd
sensitivity	14,5 μ V/mcal/sec

The thermogram, presented in Figure 13, shows three subsequent broad endotherms at respectively 60°, 77° and 117°C, which reflect the loss of 3 moles of water and one sharp exotherm at 196°C. The latter corresponds to the phase transition observed on heating a sample on a microscope hot stage in polarized light (Section 3.10.1).

In a parallel run the weight loss of 5 mg of sample was determined by weighing the DTA cup on a microbalance after a warming period (operating conditions as above). From 30 to 100°C the mean weight loss was 5,68%, and 1,93% from 100 to 130°C. The sum, (7,6 + 0,1%) is very close to the theoretical amount of water (7,50%).

3.10.3. Water Content

Natamycin, crystallized from aqueous solvents, contains approximately 7,5% of water as found by Karl Fischer titration and by loss on drying (organic solvents are not expelled)(33).

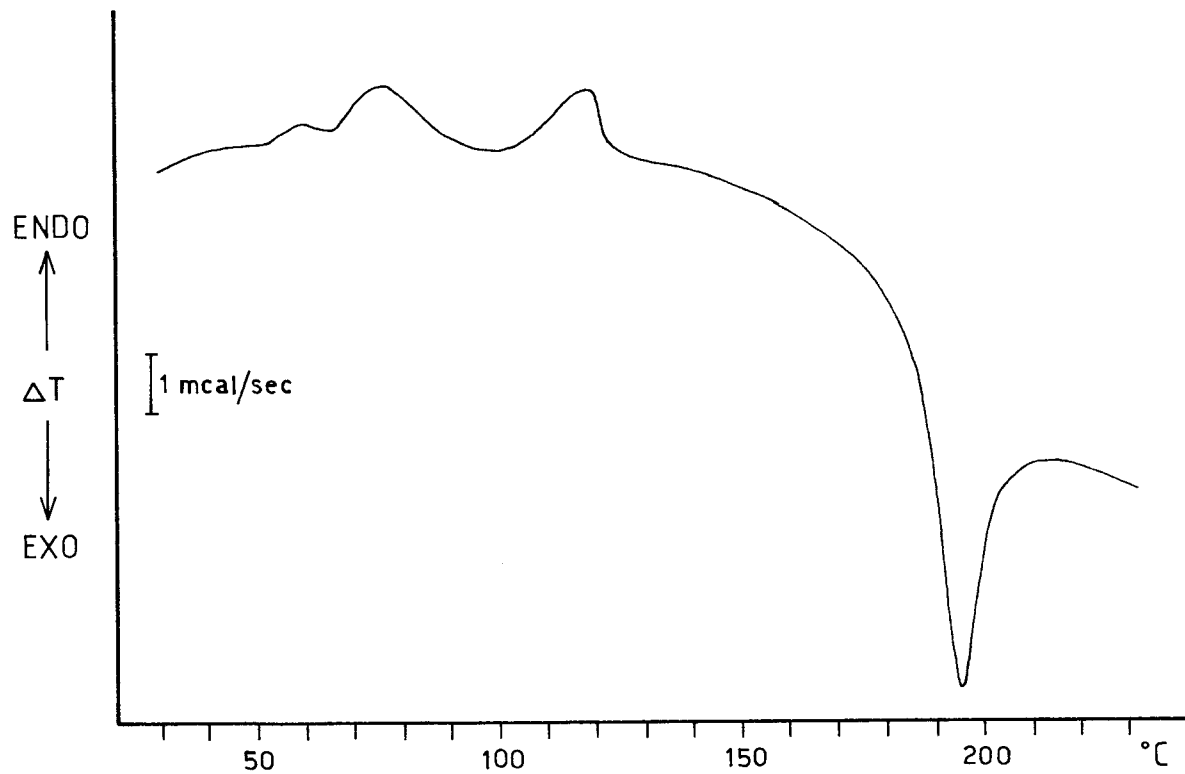


Figure 13. DTA curve of natamycin trihydrate (51)

Extremely far dried natamycin (dried in vacuo to constant weight over phosphorus pentoxide at roomtemperature), stored in the dark in air shows a weight increase of 7,45% within 4 hours, after that time the weight remains constant, see Figure 14 (33,49).

It is remarkable that only the hydrated form is chemically stable in contrast to the dehydrated substance (see Section 5).

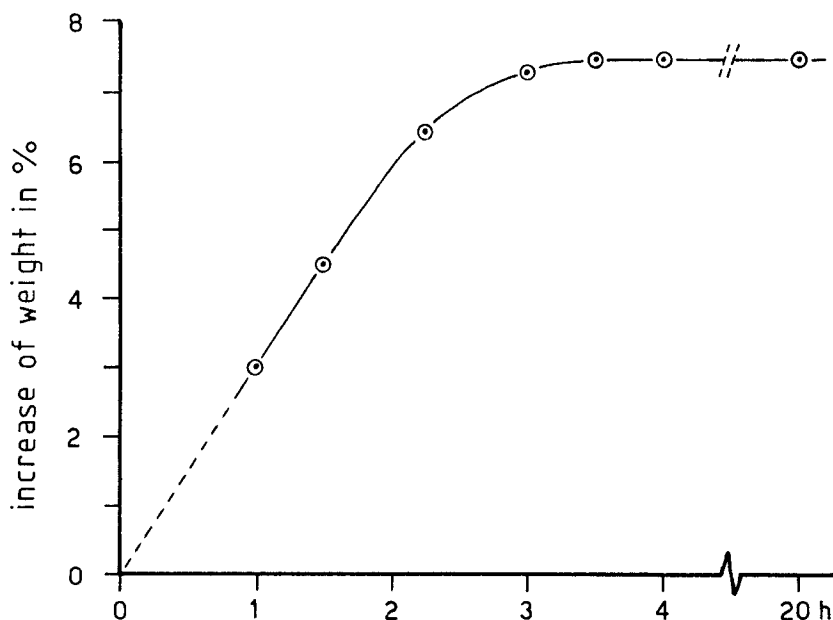


Figure 14. Absorption of water by dehydrated natamycin

3.11. Counter-current Distribution

The partition coefficient of natamycin in the system n-amylalcohol-isoamylalcohol-phosphate buffer pH 6 (12:17:29) is 1,38 after 190 transfers (52).

3.12. Solubility

Natamycin is practically insoluble in non-polar solvents. The solubility in certain polar solvents can be greatly enhanced by adding water (to for instance acetone, lower alcohols, dimethylsulphoxide) or complexing agents like calcium chloride (to methanol, methoxyethanol) or thiocyanates (to for instance acetone). The effect of water on the solubility of natamycin in dimethylsulphoxide is illustrated in Figure 15. At water contents lower than 10-15% a less soluble natamycin-dimethylsulphoxide solvate (1 to 2 moles) is formed which explains the sharp peak at 15% water of the solubility curve. Although natamycin is soluble in alkaline and acidic media the compound is rapidly decomposed in such solvents.

The solubility data, presented in Table 8, show fairly large discrepancies between different sources. Presumably this is partly caused by the formation of solvates: from saturated solutions in solvents like methanol, dioxane or methoxyethanol natamycin more or less rapidly crystallises on standing as a less-soluble solvate. For instance in methanol up to 15 mg of natamycin may dissolve per ml. However, after the spontaneous crystallization of the solvate only 3,3 mg per ml remains in solution. Further the solubility of natamycin is largely dependent on its purity. Recent batches are less soluble in a variety of solvents as compared with batches of 20 years ago which contained a much higher percentage of impurities (33). Another example of the influence of solvate formation on the solubility is given in Figure 16 which demonstrates the effect of an increasing content of chloroform on the solubility of natamycin in methanol. At concentrations of less than 50% of chloroform a crystalline natamycin-methanol solvate is rapidly formed which strongly depresses the solubility of natamycin. At concentrations of 50% of chloroform or more no solvate is formed, not even upon seeding with the methanol solvate at -20°C. At the highest concentrations of chloroform the very slight solubility of natamycin in this solvent predominates. Other effects of chloroform, i.e. upon hydrogen-bonding, may be involved.

The solubility of natamycin in dimethylformamide is difficult to estimate. At roomtemperature natamycin dissolves very slowly, after one night stirring 170 mg per ml was dissolved, the substance however was partly decomposed. Upon heating to 60°C natamycin dissolves rapidly in dimethylformamide to obtain a 25 w/v% solution without significant decomposition. In this solution however natamycin is not stable either (33).

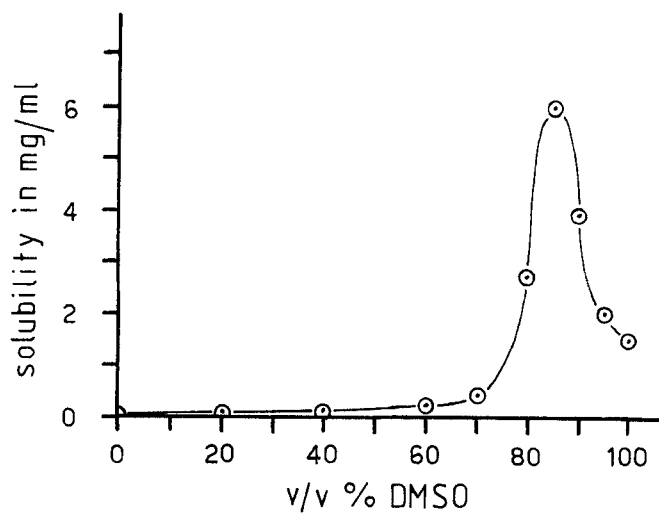


Figure 15. Solubility of natamycin in dimethylsulfoxide-water at 20°C (33).

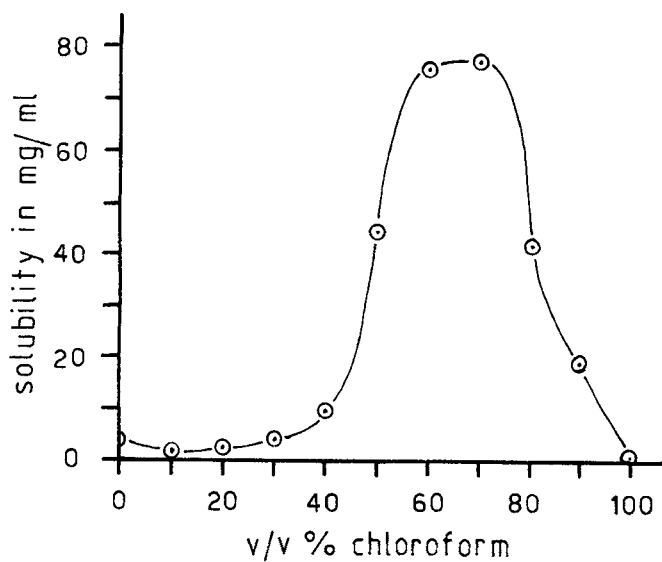


Figure 16. Solubility of natamycin in methanol-chloroform at 20°C (33).

Table 8
Solubility of natamycin in mg/ml

solvent	ref.53*	ref.33**	others
water	0,39	0,03	0,05-0,1 (38,54)
methanol	9,71	3,3	2 (38); 15 (50)
methanol + 2% CaCl ₂ ***		60	15 (38)
methanol/chloroform		see text	
ethanol	0,54	0,04	1,2 (50,54)
ethanol/water (4:1)		0,22	0,7 (50,54)
n-butanol		0,05	0,12 (50,54)
n-butanol satd. with water		0,6	1,5 (50,54)
acetone	0,073	< 0,01	
acetone/water (4:1)		0,11	
acetone + 2% KCNS		0,17	
ethylacetate	0,015	< 0,01	0,1 (54)
chloroform	0,013	< 0,01	
ethylene glycol	> 20		
propylene glycol-1,2	> 20	1,5	14 (54); 20 (38)
glycerol			15 (50,54)
formamide	> 20		20 (38)
dimethylformamide		see text	50 (38,50)
dimethylsulphoxide		see text	
methoxyethanol		1,9	
methoxyethanol + + 2% CaCl ₂ ***		15	140 (38,54)
N-methylpyrrolidone-2			120 (38,50)
glacial acetic acid		250	185 (50,54)
dioxane	0,21		
pyridine	> 20		
diethylether	0,003		

*at 21°C, calculated from evaporation residue, corrected for solvent blank.

**multiple crystallised natamycin reference standard; at 20°C after equilibration with a solvate, if any; filtrate analyzed by differential spectrophotometry.

***ref. 33: hexahydrate; ref. 38 and 54: unknown.

4. Production

4.1. Discovery

In 1955 Struyk et al. isolated a new antifungal antibiotic from a culture of *Streptomyces natalensis* nov. sp. (38). This strain was isolated from a soil sample which was taken near Pietermaritzburg, a town in the province of Natal, South Africa. The typical ultraviolet spectrum of the new antimycotic pointed to a relationship with already known polyenes like nystatin, the first member of this group which was discovered 5 years earlier.

In 1959 Burns et al. (55) isolated a compound from a culture of *Streptomyces chattanoogensis*, a strain from a soil sample of Chattanooga, Tennessee, which was called tennecetin. However, within two years this compound appeared to be identical with natamycin (39), so the name tennecetin was deleted.

A nameless tetraene, described by Backus et al. in 1959 (56), is most probably identical with natamycin. The substance was produced by *Streptomyces gilvosporeus* ATCC 13326.

4.2. Biosynthesis

The biosynthesis of the C-25 butyl homologue of natamycin, lucensomycin, has been studied using ^{14}C -labeled propionate and acetate (57). These precursors are incorporated into the aglycone. ^{14}C -labeled natamycin could be produced in the same way (58). The carbon skeleton of mycosamine is probably derived directly from glucose (59).

4.3. Fermentation and Isolation

Natamycin is produced on an industrial scale by fermentation using *Streptomyces natalensis* (60) or *Streptomyces gilvosporeus* (25). As most of the antimycotic is bound to the mycelium it is isolated either by whole broth extraction or by extraction of the mycelium, using lower alcohols (25,60). The crude compound is precipitated by pH regulation or by evaporative concentration.

5. Stability

Natamycin is a stable compound provided the powder is protected from light and moisture. Only a few percent loss of activity is observed after several years storage at roomtemperature. This is true for the trihydrate, the anhydrous form however is not stable. This form, prepared by heating the trihydrate in vacuo at roomtemperature over phosphorus pentoxide (see also Section 3.10.3), loses 15% of activity when stored for 48 hours at roomtemperature in a closed bottle in the dark (49).

Natamycin will withstand heating at up to 120°C for no more than one hour. However, any anhydrous natamycin produced during heating is unstable.

The methanol solvate (Section 3.1.1) is an unstable substance as well.

Neutral aqueous natamycin suspensions are nearly as stable as the dry powder. A neutral aqueous suspension can be boiled for a short time before a reduction in potency occurs. Aqueous solutions are quite stable at pH values between 5 and 9 if stored in the dark (54). At extreme pH values natamycin is rapidly inactivated with formation of various kinds of decomposition products (Figure 17). At a low pH the mycosamine moiety is split off. The resulting instable aglycone reacts with either a second molecule of aglycone or with a still intact molecule of natamycin. In both cases dimers with a triene rather than a tetraene group are formed. At the same time the epoxy group is hydrolysed to a diol. Heating at low pH favours decarboxylation of the aglycone (61).

At high pH values, rapidly at pH 12, the lactone is saponified with formation of the microbiologically inactive natamycoic acid (33). Treatment with strong alkali results in further disruption of the molecule owing to a series of retroaldol reactions. Among the reaction products the following compounds could be detected:

13-hydroxy-2,4,6,8,10-tetradecapentaene-1-al (1,62), acetone (4), acetaldehyde (3,4) and ammonia (1).

Natamycin is decomposed by ultraviolet radiation with loss of the tetraene structure (33,63,64). Thoma (65) observed that natamycin decomposed faster in aqueous solution at pH 4 than at pH 8 upon radiation with a xenon lamp. Visible light does not inactivate natamycin unless transfer of photo-energy by e.g. riboflavin takes place (66).

Gamma radiation decomposes natamycin as well, it can therefore not be used to sterilize the substance.

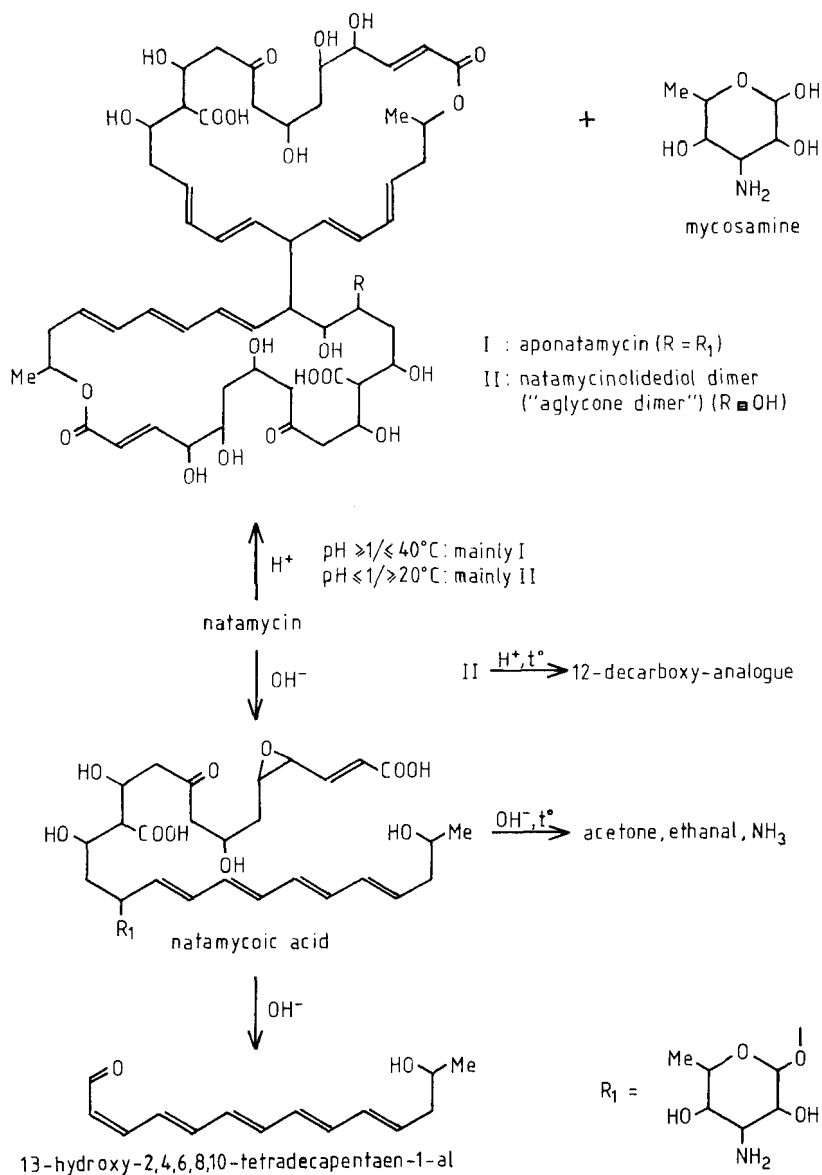


Figure 17. Decomposition of natamycin in acid and alkaline medium (I and II: tentative structure)

The inactivation by peroxides or, especially at higher temperatures, by oxygen can be prevented by antioxidants like chlorophyll, ascorbic acid (38,67,68) butylated hydroxyanisole or butylated hydroxytoluene (69).

Oxidative inactivation is promoted by several metal ions, especially Fe(III), Ni(II) and Cr(III) (33). This can be prevented by adding complexing agents like EDTA or polyphosphates (69).

Inactivation of natamycin by light, peroxides or oxygen proceeds at the fastest rate in solution or in suspension, less so in the solid form. Oxidative degradation of natamycin probably leads to the formation of polymers or compounds formed by addition of oxygen on the conjugated double bonds. The latter reaction, which takes place at one end of the polyene chain, is described for several polyenes. Either an epoxy-group (filipin and lagosin, 70) or a hydroperoxide (nystatin, 71, or levorin and mycoheptin, 72) is formed. Inactivation occurs also in the presence of sulphites or sodium formaldehyde sulfoxylate.

6. Biopharmaceutics

6.1. Pharmacokinetics

Absorption of natamycin from the human intestine after oral administration of doses from 125 to 500 mg per day during a period of 1 up to 7 days has not been observed. The serum collected did not show any antifungal activity (73). In animals the same results have been obtained. With rats and mice oral administration of natamycin only reduced the yeast count in the faeces (38,74).

6.2. Toxicity

Natamycin has a very low oral toxicity. The oral LD₅₀ in the male rat is 2,73 g/kg, in the male rabbit 1,42 g/kg (75). The chronic toxicity of natamycin was studied by the administration of natamycin in the food of rats and dogs. Only minor effects such as a slight decrease in the intake of food and a slight inhibition of growth, were observed when 1 mg per kg per day was fed to rats for two years. Dogs tolerated a dose of 0,25 mg per kg per day for more than two years, a dose of 0,5 mg per kg per day resulted in a slight decrease in body weight when administered for two years (75).

Acid degradation products (like aponatamycin, the aglycone dimer and mycosamine) and products obtained by alkaline degradation or UV radiation of natamycin are even less toxic than the parent compound (76,77).

6.3. Other

No sensitizing effect has been observed after continuous exposure to high concentrations of natamycin (78,79). This is possibly due to the low affinity of natamycin for proteins. Resistance to natamycin is not observed (80), cross resistance between natamycin and other polyenes has not been reported (81,82).

The haemolytic activity of natamycin is less than that of the more lyophilic polyenes nystatin, amphotericin B and lucensomycin (83).

Newcomer (84) reported nausea, vomiting and diarrhoea when natamycin was given orally to adults in doses exceeding 1000 mg per day.

7. Analysis

7.1. Identification

Typical colours are formed when concentrated mineral acids are added to natamycin. This reaction is based on protonation of the polyene chromophore (85). In this manner natamycin may be identified among other polyenes (33). See Table 9.

A solution of antimony trichloride in chloroform (Carr-Price reagent) gives different colours as well. Natamycin and lucensomycin give a red colour, nystatin a red-violet colour, all three shifting rapidly to dark-brown. Filipin gives a blue colour, the heptaenes listed in Table 9 colour green, shifting to blue-green (33).

Natamycin, like other polyenes, reacts with Folin-Denis reagent (molybdotungstophosphoric acid) with formation of a blue colour. This test is described in several pharmacopoeias for nystatin (86,87,88,89). The reagent is highly aspecific however since it reacts with all readily oxidizable compounds.

A somewhat more specific test is the reaction with decolourized magenta (Schiff reagent). Upon heating with several polyenes including natamycin a red colour is produced. The reaction is based on the formation of aldehydes, the heptaenes listed in Table 9 do not react. This test is also described for nystatin in a number of pharmacopoeias (86,87,88).

Table 9
Colour reaction of polyenes*

polyene	group	conc. HCl	conc. H ₂ SO ₄	conc. H ₃ PO ₄
natamycin	tetraene	brown	brown violet	red (brown)
nystatin	tetraene	brown (gray)	brown violet	red-brown (brown)
amphotericin A	tetraene	brown (gray)	brown violet	red-brown (brown)
lucensomycin	tetraene	brown- green	green brown	green (brown green)
filipin	pentaene	violet	violet	violet- brown
amphotericin B	heptaene	violet (gray)	brown	blue (violet)
candicidin	aromatic heptaene	green (brown)	blue	blue (blue- green)
levorin	aromatic heptaene	green (brown)	blue	blue (blue- green)
partricin	aromatic heptaene	green (brown)	blue	blue (blue- green)
trichomycin	aromatic heptaene	green (brown)	blue	blue (grey- ish-blue)

*in parentheses: colour after a few minutes

Natamycin may be identified by means of thin layer chromatography (Section 7.5.2), the differentiation from other common polyenes is not very clear, however.

Natamycin may be identified as a tetraene by recording the ultraviolet spectrum. By this means it can also be differentiated from the tetraenes nystatin and amphotericin A by recording down to 215 nm (90). Natamycin shows an absorption at 220 nm (en-one), nystatin and amphotericin A however show an absorption at 230 nm (trans, trans-diene).

The best methods for identification of natamycin are IR and UV spectrophotometry combined with the colour reaction with strong acids. By this means it can be differentiated from all other common polyenes.

For preparations containing significant quantities of excipients a combination of thin-layer chromatography and UV spectrophotometry is to be recommended.

7.2. Spectrophotometric analysis

Ultraviolet spectrophotometry, using methanol with 0,1% of acetic acid as the solvent, may be used for the assay of natamycin and its dosage forms. The method is useful for routine control but not for stability studies because of the noncorrelation of microbiological activity and tetraene content upon degradation. The highest degree of correlation is obtained with differential spectrophotometry (33), a principle which is also used for the assay of nystatin (91) and the heptaenes mycoheptin and levorin (92). This method is based on the measurement of absorbance at the main maximum at 303 nm and at the minima on either side, i.e. at 295 and 311 nm. From these values the base-line absorption

$$A_{303} - \frac{A_{295} + A_{311}}{2}$$

is calculated.

As seen in Tables 10 and 11 the base-line method gives more reliable results as compared with the microbiological assay than the "one point" spectrophotometric method using only absorbance measurement at the peak at 303 nm.

In both examples a 5% natamycin suspension was degraded and analyzed at specific times (33). Table 10 shows the results of degradation at pH 1,5. Under this condition mainly dimers with triene absorption though with remarkable (flank)absorption at 303 nm are formed. A small amount of inactive tetraene (aglycone) is responsible for the base-line method yielding too high results. Table 11 shows the results of degradation by long-wave ultraviolet radiation. In this way no inactive tetraene is formed so the results of the base-line method are nearly equal to those of the microbiological assay.

When natamycin is degraded in alkaline medium a compound is formed which strongly interferes with the base-line method. For instance, a 5% aqueous solution of natamycin at pH 12 was totally inactivated within a few hours, spectrophotometrically however, using the base-line method, no decrease in tetraene content could be detected (33). This can be readily explained by the formation of the microbiologically inactive tetraene natamyoic acid, formed by simple saponification of natamycin (see Section 5).

Table 10
Degradation of natamycin at pH 1,5

% of natamycin left		
one-point method	base-line method	bio-assay
100	100	100
81	74	75
59	50	46
39	31	14
23	14	2
21	5	< 0,5

Table 11
Degradation of natamycin by UV light*

% of natamycin left		
one-point method	base-line method	bio-assay
100	100	100
83	78	79
67	62	60
46	40	39
32	22	20
8	6	5

*unfiltered, low pressure mercury lamp

7.3. Colorimetric Analysis

Natamycin shows a transient blue colour in fairly strong hydrochloric acid, owing to the formation of a carbonium ion (85). This principle was used by Dryon (93) to perform a colorimetric determination of natamycin. To four volumes of a methanolic solution of natamycin containing 30 to 190 μg per ml are added ten volumes of concentrated hydrochloric acid containing 20% of ethanol under cooling with ice. After 13 - 15 minutes the absorbance is measured at 635 nm. The blue colour does not obey Beer's law.

A number of acid and alkaline degradation products of natamycin does not interfere in this method (33).

A more sensitive determination (4 - 20 μg of natamycin per ml of resulting solution) is based on the formation of a red chromophore on treatment of an alkaline hydrolysis product of natamycin (13-hydroxy-2,4,6,8,10-tetradecapentaene-1-al) with 4-aminoacetophenone (33), analogous to a colorimetric determination of nystatin described by Amer et al. (94). Beer's law is obeyed within the above concentration range. Sugars and some alkali-inactivated decomposition products of natamycin interfere.

7.4. Titrimetric Analysis

Natamycin can be titrated potentiometrically with perchloric acid in non-aqueous media like glacial acetic acid. Analytically however this method is of little value. Titration in partially aqueous media has been used for determination of the ionisation constants.

Interesting is the titration of natamycin with kation tensides where the formation of the ion-pair is followed by conductimetry (33). Figure 18 shows the titration curves of natamycin (dissolved with the aid of an equivalent amount of sodium hydroxide, 0,1 mmol in this case) and a prominent anion tenside like sodium laurylsulphate (both 0,1 mmol in 25 ml of water) with 0,02M cetyltrimethylammonium bromide. Natamycin clearly behaves as an anion tenside.

7.5. Chromatographic Analysis

7.5.1. Paper Chromatography

Several systems for paper chromatography of natamycin on Whatman no 1 paper are listed in Table 12. Spots were detected bioautographically.

Table 12
Paper chromatographic systems for natamycin

Solvent system	R _f value	Reference
n-butanol/water, saturated	0,33	55
n-butanol/ethanol/water (5:1:4)	*	38
n-propanol/water (7:3)	*	38
triethylamine/formamide/water (10:3:10), upper layer	0,33**	25

*not reported, but separation from three other tetraenes possible

**relative to chromin

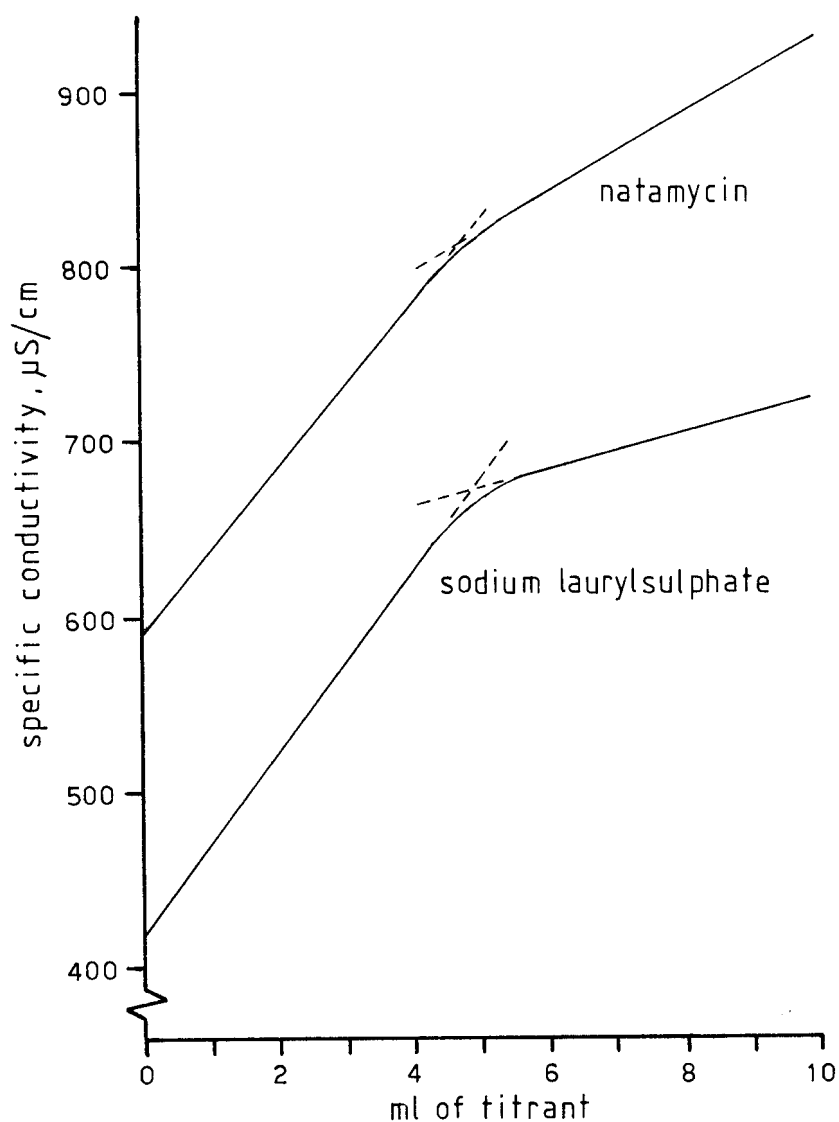


Figure 18. Conductometric titration of natamycin and sodium laurylsulphate (each 0,1 mmol) with 0,02M cetyltrimethylammonium bromide.

7.5.2 Thin Layer Chromatography

Thin layer chromatography has been used in qualitative analysis to differentiate the antibiotic from other polyenes or to test its purity. Several systems are listed in the Tables 13, 14 and 15.

In testing the stability solvent systems 10 and 13 are useful to estimate mycosamine in natamycin and its preparations. Ninhydrine is used as the detectant. Afterwards the same plate can be sprayed with a universal detectant like sulphuric acid to detect aglycone-like degradation products (33,61).

Table 13
Thin-layer chromatography systems for natamycin

stationary phase	solvent system (Table 14)	method of detection (Table 15)	R _f value	Ref.
Silicagel G (Merck) (pH 8)	1	1	0,34	95
Silicagel G (Merck)	2	1	0,34	95
Silicagel G (Merck)	2	2,3	0,57	96
Silicagel G (Merck)	3	4	0,40	93
Silicagel G (Merck)	4	4	0,54	93
Silicagel G (Merck)	5	2,3	0,18	96
Silicagel G (Merck)	6	2,3	0,55	96
Silicagel G (Merck)	7	2,3	0,75	96
Silicagel G (Merck)	8	2,3	0,60	96
Silicagel G (Merck) (pH 3)	9	5	0,59	97
Silicagel 60 F 254 (Merck)	10	6,10	0,4	61
Silicagel 60 F 254 (Merck)	11	7,8	0,33	45
Silicagel GF (Analtech)	12	7,8	0,45	45
Polygram Sil G foil (M and N)	13	9,10	0,7	33
Sephadex G-15	14	11	0,7*	98

*relative to benzylpenicillin

Table 14Thin-layer chromatography systems for natamycin
Solvent systems

1. ethanol/ammonia/water (8:1:1)
2. n-butanol/acetic acid/water (3:1:1)
3. methanol/isopropanol/acetic acid (90:10:1)
4. methanol/acetone/acetic acid (8:1:1)
5. ethanol/ammonia/dioxane/water (8:1:1:1)
6. n-butanol/pyridine/water (3:2:1)
7. n-butanol/pyridine/acetic acid/water (15:10:3:12)
8. n-butanol/acetic acid/water/dioxane (6:2:2:1)
9. n-butanol/acetic acid/water (2:1:1)
10. chloroform/methanol/acetic acid/water (6:2:2:1)
11. chloroform/methanol/0,05M borate buffer pH 8,3 (2:2:1), lower layer
12. n-butanol/acetic acid/water (4:1:5), upper layer
13. n-butanol/acetic acid/water (4:1:2)
14. 0,025M phosphate buffer pH 6,0 containing 0,5M NaCl

Table 15Thin-layer chromatography systems for natamycin
Methods of detection

1. 10% potassium permanganate/0,2% bromophenolblue
2. 5% potassium permanganate
3. concentrated phosphoric acid, 5 minutes at 100°C
4. 0,2% p-dimethylaminobenzaldehyde in concentrated sulphuric acid containing a trace of ferric chloride
5. 1% p-dimethylaminobenzaldehyde + 20% antimony trichloride in ethanol with 20 v/v% concentrated hydrochloric acid
6. concentrated sulphuric acid, 10 minutes at 105°C
7. iodine vapour
8. concentrated sulphuric acid/glacial acetic acid (1:1)
9. concentrated sulphuric acid/methanol (1:2)
10. ninhydrine
11. bioautography

7.5.3 High Pressure Liquid chromatography

HPLC has been used by Frede (99) for the identification of natamycin in cheese-extracts. The detection limit was 20 ng per injection at a detection wavelength of 303 nm. As HPLC is much more selective than the UV spectrophotometric method it is a useful method to assay partially degraded samples, pharmaceutical dosage forms or biological material. Several systems are listed in Table 16. A chromatogram of the USP reference standard is shown in Figure 19.

Table 16
Systems for HPLC of natamycin

Stationary phase	Eluent	Ref.
Lichrosorb RP-8 25 cm (Merck)	MeOH-H ₂ O (65 : 35)	99
μBondapak C18 25 cm (Waters)	MeOH-H ₂ O-HOAc (48 : 32 : 1)	100
μBondapak C18 25 cm (Waters)	MeOH-H ₂ O-THF (44 : 47 : 2) containing 1 w/v% of NH ₄ OAc	101

7.6. Electrophoretic Analysis

Ochab (102) separated natamycin from several other polyenes by means of electrophoresis on Whatman no. 4 and no. 34 paper, mobilities in four different electrolytes are reported.

7.7. Polarographic Analysis

Dornberger (103) determined natamycin and its C₂₅ butyl homologue lucensomycin polarographically at the dropping mercury electrode in 0,2M phosphate buffer pH 7 in a concentration range of 10⁻⁴ to 10⁻⁵M.

The epoxy group of natamycin is reduced at a half-wave potential of -0,85 V versus the normal calomel electrode. Lucensomycin gives a wave at -1,0 V. Derivatives or polyenes which lack an epoxy group react negatively.

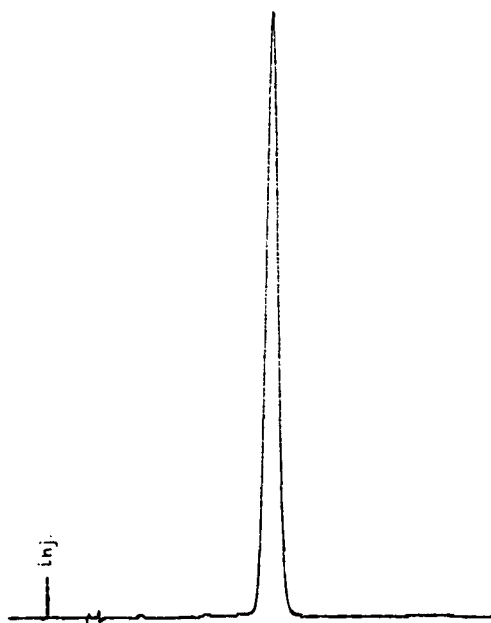


Figure 19. High-pressure liquid chromatogram of 4 μ g of natamycin USP reference standard (104)

Instrument	: Spectra Physics SP 8000 chromatograph with a Schoeffel SF770 detector
Column	: μ Bondapak C18 3,9 x 300 mm
Mobile phase	: methanol-distilled water-tetrahydrofuran (440 : 470 : 20) containing 1% of ammonium acetate. Rate of flow: 2 ml/minute.
Detection	: Ultraviolet absorption at 303 nm
Sensitivity	: 0,04 AUFS
Retention time	: 13,5 minutes

7.8. Elemental Analysis

The presence of ash, organic impurities (especially in earlier lots), solvent of crystallization (i.e. methanol, water) may bring about substantial lack of agreement between older experimental data and the recent theoretical composition. Possibly rather because of the presence of the above foreign constituents there was sometimes a fairly good agreement between experimental and -meanwhile obsolete- theoretical data (1,62). Recent experimental data (49), obtained with natamycin trihydrate reference standards, conform very well with modern theoretical data.

Table 17
Elemental analysis of natamycin

theoretical composition in %				
	C	H	N	O
natamycin anhydrous	59,54	7,12	2,10	31,24
natamycin trihydrate	55,06	7,42	1,95	35,57

found in %

C	H	N	O	ref.	remarks
58,53	7,32	2,12	-	1	mean value of seventeen analyses in one sample (1958)
57,11	7,33	2,08	-	62	mean value of the analysis of seven recrystallized samples (1964)
55,11	7,41	1,99	34,98	49	mean value of the analysis of three specially prepared reference standards (1973-1976)

7.9. Microbiological Analysis

Natamycin is assayed microbiologically with *Saccharomyces cerevisiae* ATCC 9763 as the test organism using the agar diffusion method. The assay is recommended for the determination of natamycin in solutions or extracts of the substance, its dosage forms or in biological material. The sensitivity of the agar diffusion method is approximately 0,5 µg per ml of solution (105).

An interesting alternative for the bioassay of natamycin is based on measurement of the decrease in heat output rate with time of the respiration of *Saccharomyces cerevisiae* (106). The determination, which proceeds by flow microcalorimetry, was carried out in a concentration range of 1 to 7×10^{-6} M.

8. Acknowledgment

The author thanks Dr J. de Flines, Dr H.J. Kooreman, Dr A.P. Morgenstern, Dr D.A.Smink and Ir J.A. van der Straaten for reviewing the manuscript, Drs G.J.B. Corts and Drs C. van der Vlies for their valuable suggestions for improvements, the many contributors cited as "personal communication" and Ir J.C. Monshouwer for his technical assistance in preparing the manuscript.

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Literature surveyed through February 1981.

OXYTOCIN

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1. Description

Oxytocin is the cyclic octapeptide⁺⁾ hormone released by the posterior pituitary and having uterotonic and galactagenic activity in mammals and hypotensive activity in birds.

Its 20-membered ring is composed of five amino acids - cystine, tyrosine, isoleucine, glutamine and asparagine -, and the side chain contains a further 3 amino acids - proline, **leucine and glycineamide**. All the optically active amino acids belong to the L-series.

The structure of oxytocin was elucidated by du Vigneaud *et al.*, and independently by Tuppy in 1953 (1,2). The structure was confirmed by du Vigneaud *et al.* by synthesis shortly afterwards (3).

1.1 Nomenclature

1.11 Chemical names

L-Cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyll-L-asparaginyll-L-cysteinyl-L-prolyl-L-leucyl-glycinamide cyclic (1 \longrightarrow 6) disulphide

L-Hemi-cystinyl-L-tyrosyl-L-isoleucyl-L-glutaminyll-L-asparaginyll-L-hemi-cystinyl-L-prolyl-L-leucyl-glycinamide

1.12 Generic name

Oxytocin [50-56-6]

1.13 Brand names

The following brand names are listed in the Merck Index (4): Alpha-hypophamine; Ocytocin; Endopituitrina; Pitocin; Syntocinon; Nobitocin S; Orasthin; Oxystin; Partocon; Synpitan; Piton-S; Uteracon.

1.2 Formula

1.21 Amino acid sequence

1	2	3	4	5	6	7	8	9
Cys	Tyr	Ile	Gln	Asn	Cys	Pro	Leu	Gly-NH ₂

Cys-Tyr-Ile-Gln-Asn-Cys

⁺⁾ Oxytocin is also known as a nonapeptide in the literature. Instead of 1 cystine 2 cysteines can be used for the characterization of the amino acid sequence (see 1.1).

side chain is bent back to the ring.

Figure 1.1 is a diagram of the proposed configuration.

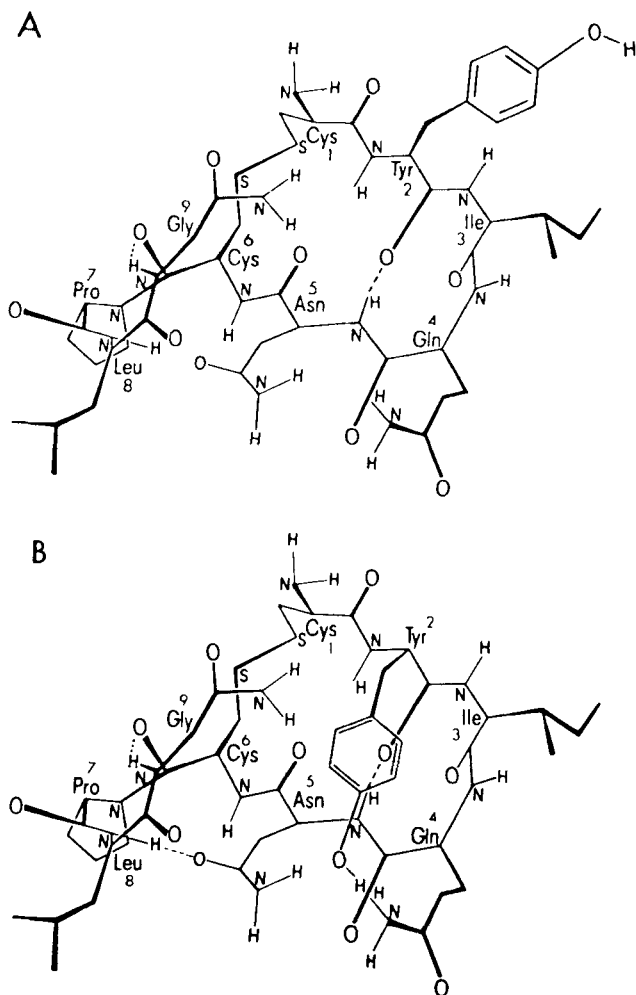


Figure 1.1

Proposed conformations of oxytocin in solution as published by Gross and Meienhofer (6).

A: in dimethylsulphoxide

B: at receptor sites

1.6 Appearance, colour, odour

The free base has not been obtained in crystalline form. By freeze-drying solutions of oxytocin acidified with acetic acid the acetate is obtained, a white fluffy powder with a faint odour of acetic acid.

1.7 Biological activity

Oxytocin is difficult to prepare in a pure form owing to the complex synthesis. Different biological activities have been reported in the literature for allegedly pure oxytocin, thus suggesting that the preparations in question differed in purity. Chan and du Vigneaud reported an activity of 507 ± 23 units/mg (7). Maxfield and Scheraga worked with oxytocin with an activity of 495 ± 25 units/mg (8). A similar activity, i.e. approximately 500 units/mg, was reported by Glickson et al. (9) and by Cerletti and Berde (10), while Deslauriers et al. described a compound having an activity of 510 ± 23 units/mg (11). Boissonnas and Huguenin reported an activity of 450 ± 30 units/mg (12), and Photaki an activity of approximately 400 units/mg (13). Bockaert et al. described a ^3H -oxytocin with an activity of 440 units/mg (14). The National Institute of Standards and Control (London, UK) indicates an activity of 595 units/mg for the Fourth International Oxytocin Standard, a synthetically prepared and specially purified product.

Oxytocin acetate was purified by preparative column chromatography on silica gel and the eluate was freeze-dried⁺. The product so obtained had a biological activity determined by the rat uterus method (15) of 591 ± 23 units/mg. If allowance is made for nonpeptide impurities (4.3% water, 8.7% acetic acid and 0.5% sodium), the activity is 684 ± 27 units/mg peptide. The compound was shown by thin-layer chromatography (stationary phase: silica gel; solvent system: chloroform-methanol 7:3 + 5% 0.2 N acetic acid) to be homogeneous, while HPLC (see section 6.27) showed it to contain less than 1% of detectable and presumably peptide byproducts.

2. Physical properties

2.1. Infrared spectrum

The Infrared spectrum of the compound described in section 1.7 was recorded from 4000 to 600 cm^{-1} using a Beckman Acculab

⁺) We are grateful to Mr. H. Bossert, Sandoz Ltd., for preparing the purified active compound.

8 apparatus. The spectrum of a KBr pellet prepared with 1.5 g active compound and 300 mg KBr is shown in Figure 2.1. Some regions of the spectrum differ from the catalogues of spectra (16). The differences are attributable to the varying degrees of purity of the compounds employed.

2.2 Ultraviolet absorption

The UV spectrum (Figure 2.2) of the product described in 1.7 was recorded for an aqueous solution with a concentration of 0.3 mg/ml over the range 210 - 320 nm using a Pye-Unicam apparatus, Type SP 1700. The absorption maximum was at 275 nm and there was a shoulder at 281 nm. After correcting for non-peptide impurities which do not absorb UV light at 275 nm, the $E_{1\text{cm}}^{1\%}$ value is 14.9 and the molar extinction coefficient is 1500. The spectrum is in good agreement with the literature (16), but the molar extinction coefficient is higher than that reported in the literature, owing to the greater purity of the compound (16).

2.3 Circular dichroism

CD spectra for oxytocin have been described in the literature by various authors (15,18). Beychok and Breslow investigated the CD spectrum at various pH values of an oxytocin preparation with an activity of 500 units/mg (17). The spectra obtained are shown in Figure 2.3. The characteristic range of wavelengths is 215-310 nm.

At acid pH values oxytocin displays a negative band at 280 nm, a positive shoulder at 250 nm and a large positive band at 225 nm. When the solution is neutralised (pH = 7.5) the negative band declines in intensity, the shoulder at 250nm becomes a true maximum and the band at 225 nm undergoes a shift to somewhat longer wavelengths. At pH 10.6 (ionisation of tyrosine) a striking change occurs: a positive plateau appears at 280 - 290 nm and there is a large positive band at 245 nm. The optical activity at 280 nm is contributed by tyrosine and the disulphide bond, while the optical activity in the region of 225 nm is attributable to transition of tyrosine to the ionised state.

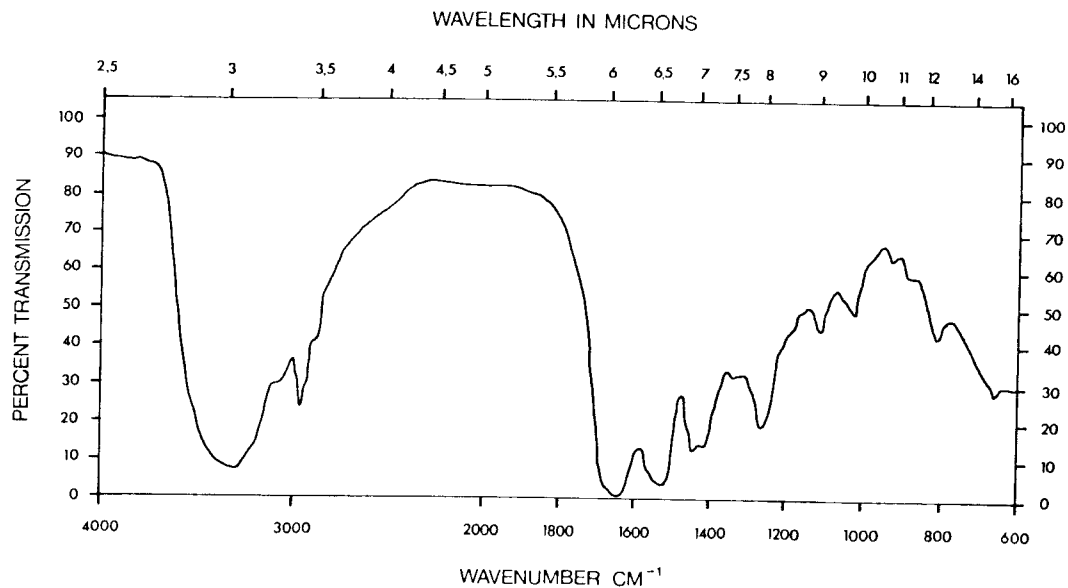


Figure 2.1

Infra-red spectrum of oxytocin, activity 591 ± 23 units/mg.
KBr pellet; spectrometer: Beckman Acculab 8

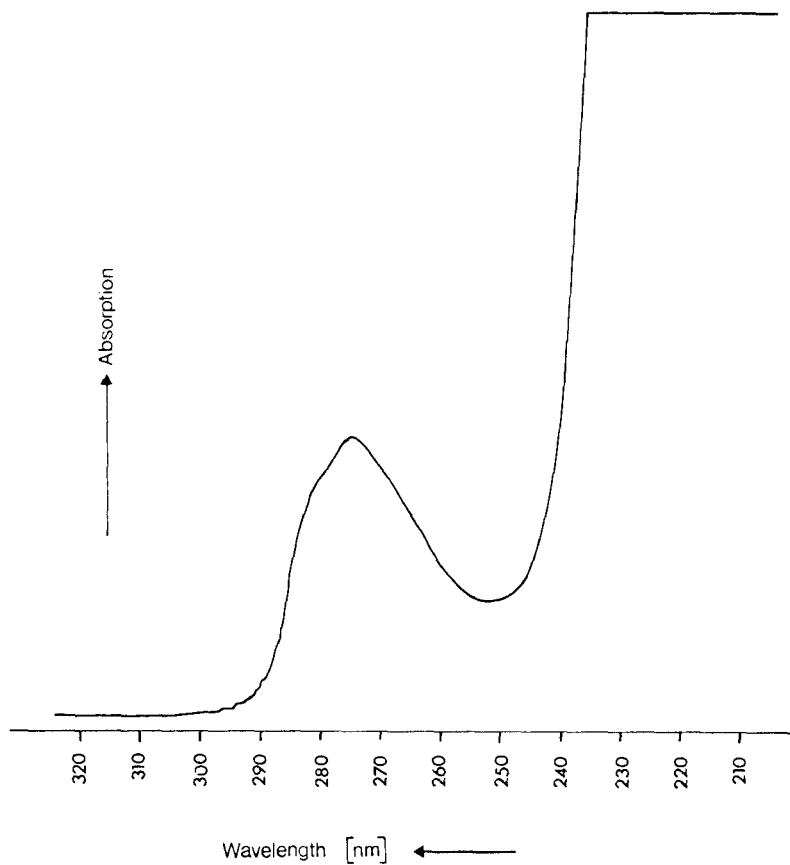


Figure 2.2

UV spectrum of oxytocin, activity 591 ± 23 units/mg
Concentration, 0.3 mg/ml H_2O
Spectrometer: Pye Unicam SP 1700

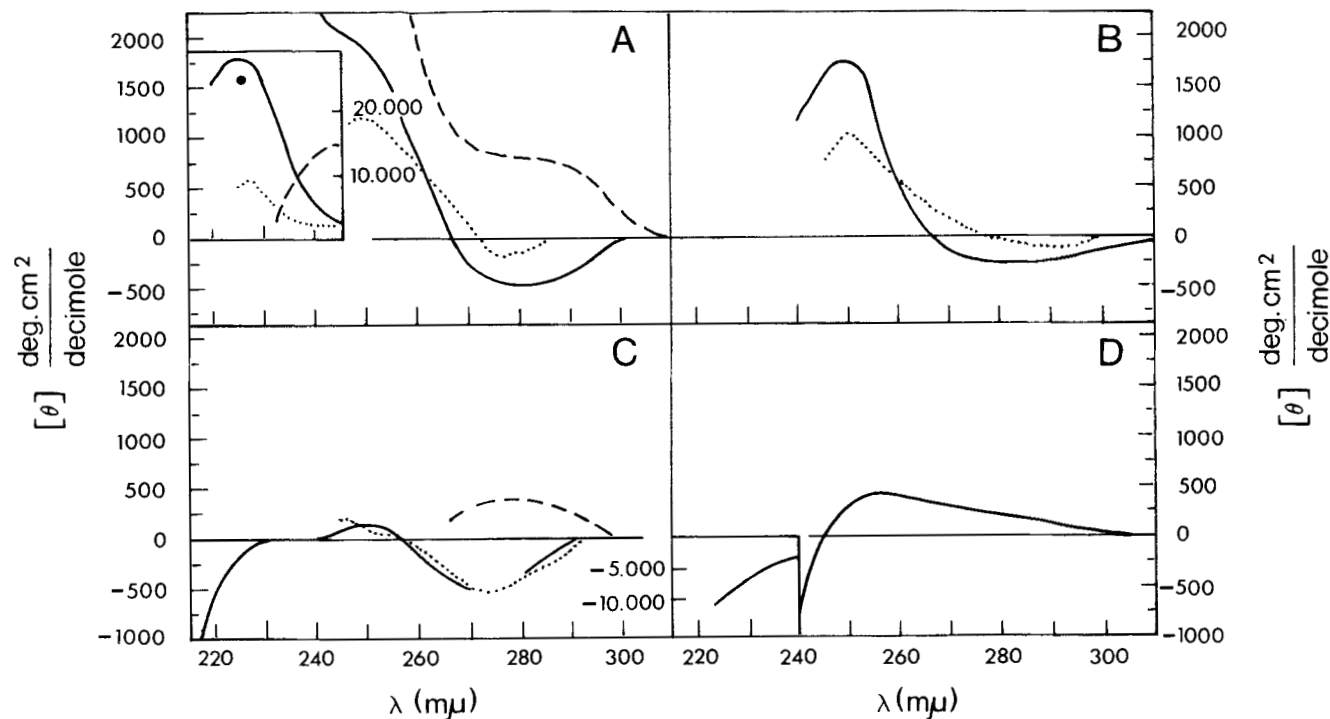


Figure 2.3

CD spectra of oxytocin (activity 500 units/mg) and oxytocin analogues at various pH values; published by Beychok and Breslow (17)

2.4 Raman spectra

Investigations of the Raman spectra of oxytocin have been described in the literature (8,19). Raman spectra are shown in Figures 2.4 and 2.5. The incident radiation was the 514.5 nm line of an argon ion laser (Spectrophysics, Model SP-164), and the spectra were recorded with a Spex Ramalog 5.

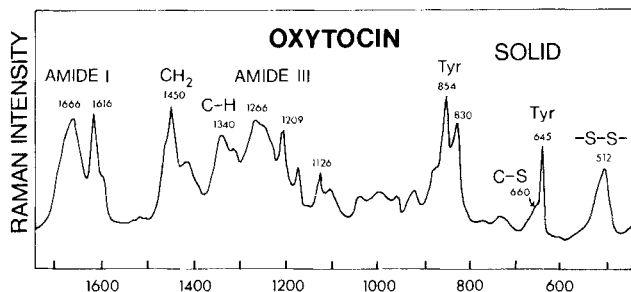


Figure 2.4

Laser-Raman spectrum of oxytocin in the solid state

Incident light: 150 mW, resolution: 5 cm⁻¹, integration time 2s

Scan rate: 6 cm⁻¹/min; published by Tu et al. (19).

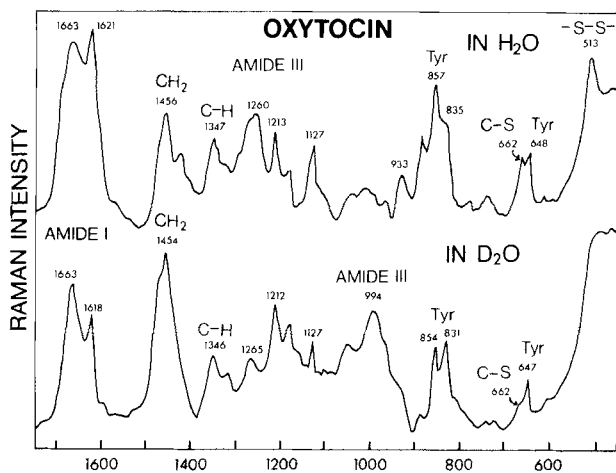


Figure 2.5

Laser Raman spectrum of oxytocin in aqueous solution and in D₂O

Incident light: 500 mW, other conditions as in Figure 2.4;

published by Tu et al. (19).

2.5 Proton NMR

The spectrum of the compound described in 1.7 was recorded at 360 MHz with a Bruker WH-360 spectrometer (Figure 2.6)⁺⁾. 5 mg oxytocin was dissolved in 400 μ l d_6 -DMSO and one drop of mixture of $CDCl_3$ and TMS was added to the solution. This accounts for the small signal for $CHCl_3$ at 8.31 ppm. TMS, $\delta = 0$ ppm, was used as the internal standard. The spectrum is in good agreement with the data in the literature (9,20). The 1H -NMR spectrum of oxytocin has been discussed in detail by Glickson et al. (9). All the amino acids were unequivocally assigned to the spectral features.

2.6 ^{13}C -NMR

The spectrum (Figure 2.7) was recorded at 90.5 MHz with a Bruker WH-360 spectrometer.⁺⁾ 100 mg of the compound described in 1.7 was dissolved in 2.5 ml D_2O and the pD was adjusted to 3.6 with CH_3COOH . Dioxane, $\delta = 67.8$ ppm, was used as the internal standard. The spectrum is in very good agreement with spectra published in the literature. For the assignment of the signals and interpretation of spectrum, the reader is referred to papers by a number of authors (11,21-25). All the amino acids were unequivocally assigned to the spectral features.

2.7 Solubility

The solubility of the compound described in 1.7 (freeze-dried oxytocin as the acetate) was determined in three solvents. The saturated solutions were assayed by HPLC (see 6.27), and the results are shown in Table 2.1.

Table 2.1

Solubility of oxytocin (as the acetate)

Solvent	Units/ml
Water	37 800
Methanol	86 400
Dichloromethane	2.9

^{+) We are grateful to Mr. M. Loosli, Sandoz Ltd., for recording the spectrum.}

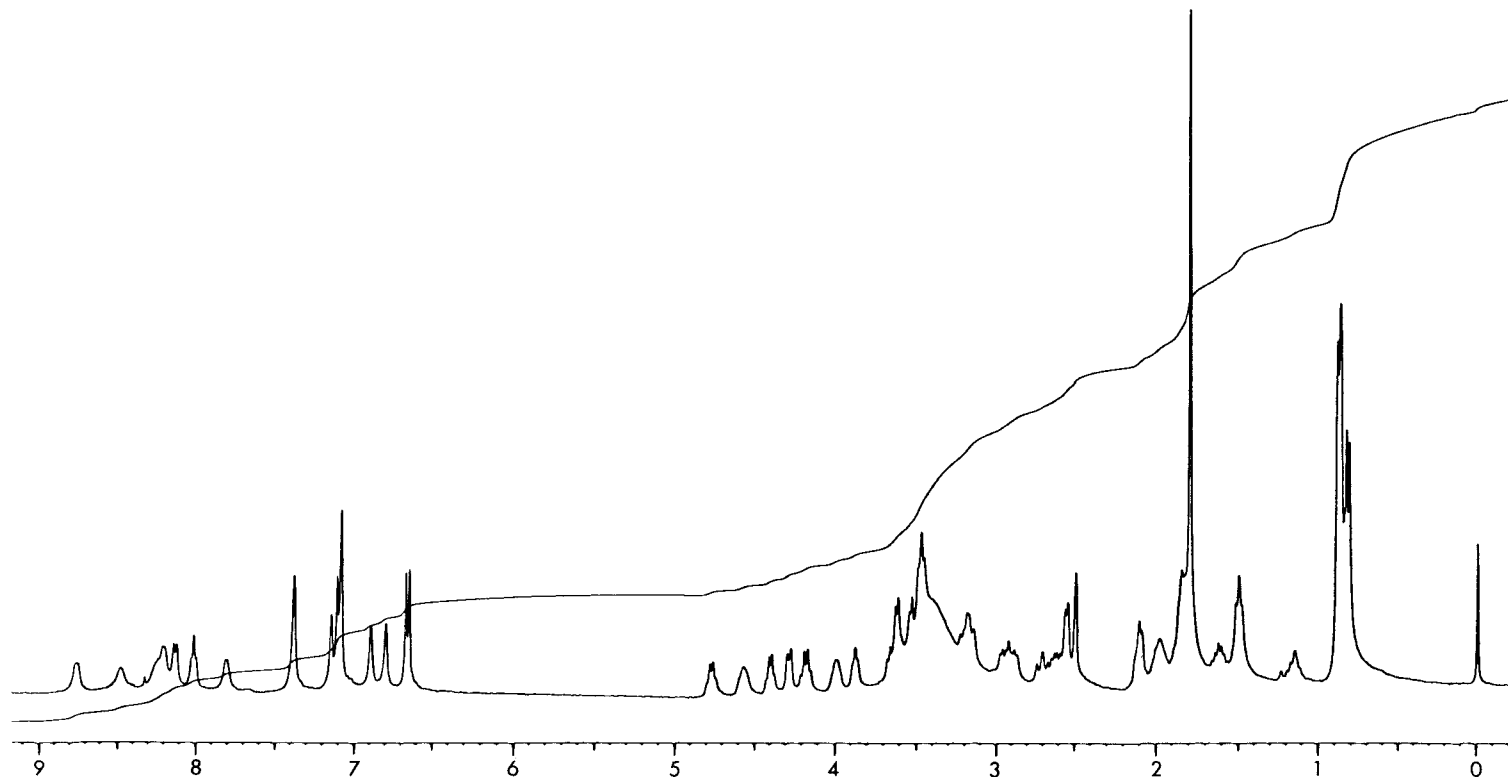


Figure 2.6 ^1H -NMR-Spectrum of oxytocin, activity 591 ± 23 units/mg, in d_6 -DMSO
Apparatus: Bruker WH-360

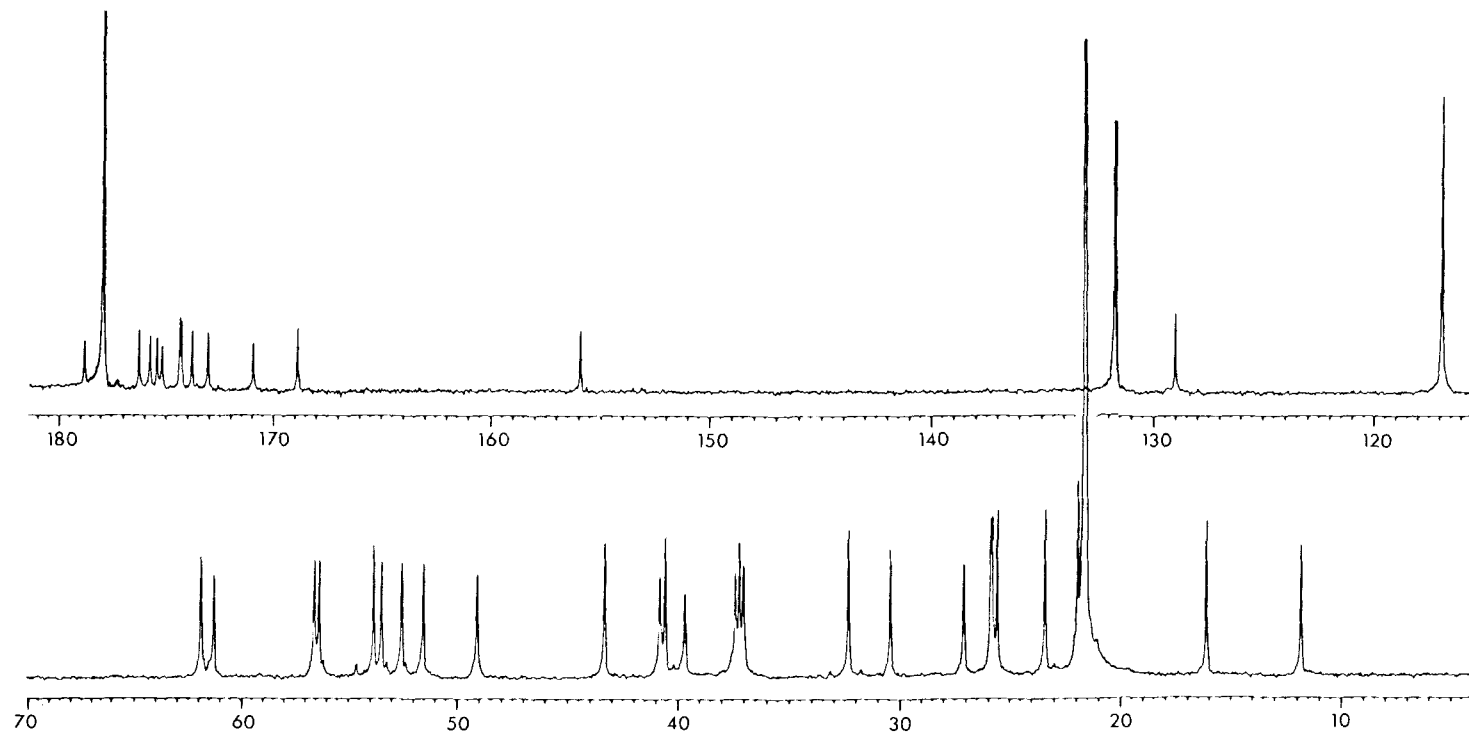


Figure 2.7 ^{13}C -NMR-spectrum of oxytocin, activity 591 ± 23 units/mg, D_2O (pL = 3.6)
Apparatus: Bruker WH-360

2.8 Optical rotation

Optical rotation values for oxytocin from the literature are given in Table 2.2.

Table 2.2

Optical rotation of oxytocin

α	Value	Conditions	Reference
$[\alpha]_{\text{D}}^{21.5}$	$-26.1 \pm 1,0^\circ$	$c = 0,53$; water	26
$[\alpha]_{\text{D}}^{22}$	-26.2° ⁺⁾	$c = 0.53$; water	26
$[\alpha]_{\text{D}}^{24}$	-24.0°	$c = 0.5$; 1 N acetic acid	13
$[\alpha]_{\text{D}}^{22.5}$	-23.1°	$c = 0.51$; 1 N acetic acid	27
$[\alpha]_{\text{D}}^{21.5}$ $[\alpha]_{\text{D}}^{-22.5}$	-23°		28

⁺⁾ natural oxytocin

2.9 Isoelectric point

Oxytocin is an amphoteric compound. Accordingly, the isoelectric point reported in the literature is at pH 7.7 (26,28, 29), consistent with the presence in the molecule of a free amino group and a free phenol group.

3. Production

3.1 Extraction from gland material

Today the extraction of oxytocin from posterior pituitary gland material is of little practical importance and largely of historical interest.

The first experiments with hypophyseal extracts were carried out by Oliver and Schäfer and date back to 1895 (30). At that time the extracts contained the pressor principle vasopressin as well as oxytocin. Preparations having both oxytocic and pressor activity were known as pituitrin and were

employed in medicine.

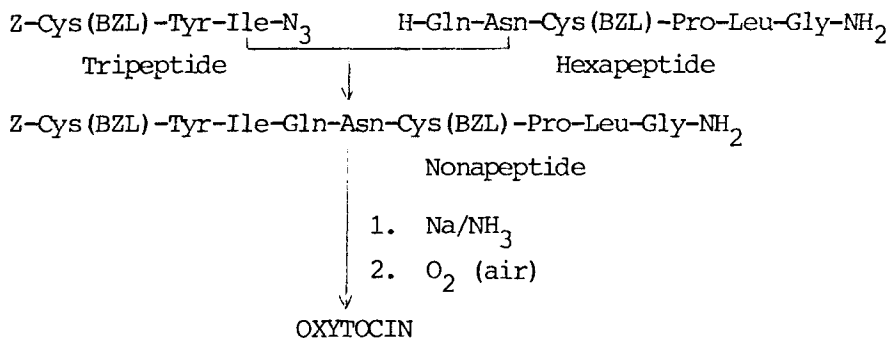
Kamm (31) described a method of preparing hypophyseal extracts without pressor activity; the gland material is dried with acetone and extracted with hot 0.25% acetic acid, and a crude product is salted out from the concentrated extracts with ammonium sulphate. This product is extracted with acetic acid, and the active material consisting of equal parts of oxytocin and vasopressin is precipitated by addition of a mixture ether/petroleum ether. The two components are separated by exploiting their different solubilities in organic solvents. Ether is added to an acetic acid solution of the active material to precipitate the vasopressin which is filtered off. Oxytocin is obtained as a solid substance with a rubbery consistency by addition of a little water and petroleum ether. The pressor effect of the hormone so obtained is only 3 - 4% of its oxytocic activity.

3.2 Chemical synthesis

The first synthesis by du Vigneaud et al. (3,32) was followed by further syntheses within the next years (33-36) differing in the protective groups used, the peptide linkage methods employed and the plan followed in building up the molecule.

The protective groups which have been mainly employed in the large-scale production of oxytocin are tosyl, carbobenzoxy and t-butyloxycarbonyl residues for the amino group and the benzyl residue for the mercapto group. The methods which have proved of value for effecting peptide linkage are the mixed anhydrides method, the active esters method using p-nitrophenol, hydroxysuccinimide or hydroxybenztriazole in combination with dicyclohexylcarbodiimide, and the azide method. The open chain N- and S-protected nonapeptide which is the precursor of oxytocin may be constructed on the 6 + 3 or the (5 + 2) + 2 plan, these intermediates being synthesised step by step.

In the method employing tosyl and carbobenzoxy residues, the last stages consist in cleavage of the protective groups with sodium in liquid ammonia followed by oxidation with air to close the ring, yielding oxytocin. As an example of a 3 + 6 plan, the synthesis according to Boissonnas (33) is outlined in the following scheme.



4. Stability

Freeze-dried oxytocin acetate may be kept in a refrigerator (2 - 6°C) without special precautions for several years with no significant loss of oxytocic activity (37). However, Ressler and Popenoe (28) mention that inactivation may occur by disulphide interchange. The shelf life of aqueous solutions is greatly dependent on the pH (Figure 4.1).

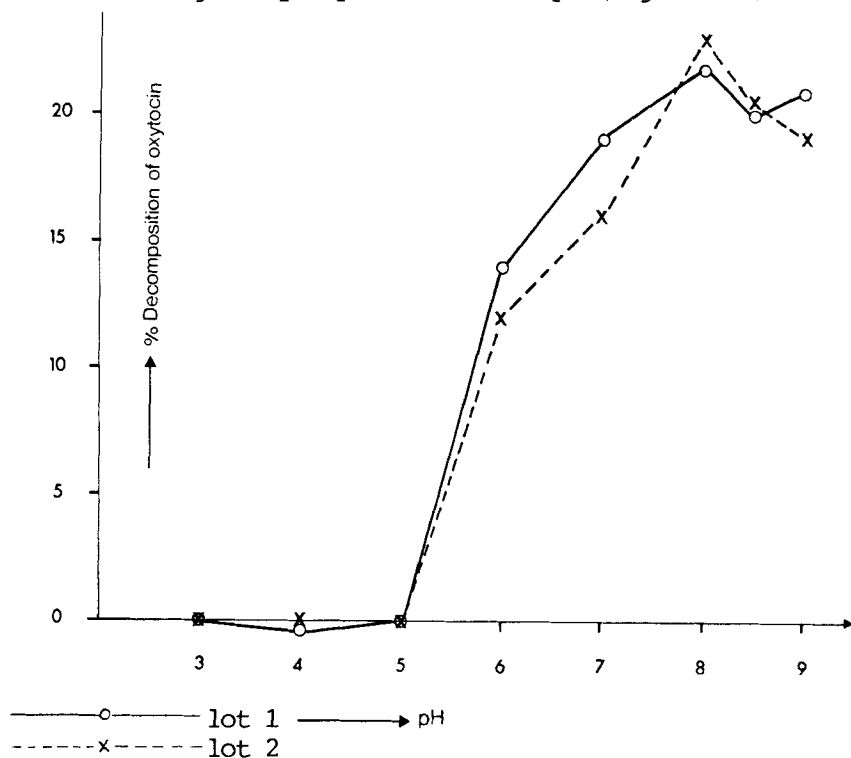


Figure 4.1

Loss of activity as a function of pH of oxytocin solutions containing 200 units/ml which had been boiled for 30 minutes.

Figure 4.1 shows the residual content of oxytocin, assayed by HPLC, in solutions at different pH values, which had been boiled for 30 minutes (38).

The optimum pH range is 3 - 5. In strongly acid solutions the peptide linkages undergo hydrolysis. Under neutral and weakly alkaline conditions, dimeric and polymeric compounds are formed, especially in concentrated solutions, by conversion of the intramolecular disulphide bridges of two or more oxytocin monomers to intermolecular bridges (disulphide interchange) (28).

A sterile aqueous concentrate of oxytocin at optimum pH, containing a preservative, will keep for several years in a refrigerator. Figure 4.2 shows the oxytocic activity (rat uterus) of oxytocin concentrate which had been stored at various temperatures.

The concentrate contained 200 units oxytocin/ml in a sterile solution at pH 3.5, containing trichlorobutanol (39). Concentrates which had been kept in the refrigerator showed no loss of oxytocin activity. Concentrates kept at 21°C showed a slight loss of activity (approx. 1.5% per year), whereas concentrates kept at 30°C showed a marked loss of activity (approx. 10% per year). This indicates that the concentrate has a shelf life of at least 3 years even at 21°C, but should not be exposed to higher temperatures.

Similar results were obtained with dilute injections of oxytocin (37). In line with these findings, some pharmacopoeias⁺⁾ specify the following shelf lives for oxytocin injection:

Eur. P.:	2 years at 25°C
B.P. 73:	at least 3 years at 2 - 10°C
Compendium	
Medicamentorum:	5 years at 15°C

⁺⁾ Abbreviations of the pharmacopoeias as in Martindale, The Extra Pharmacopoeia, 27th Edition

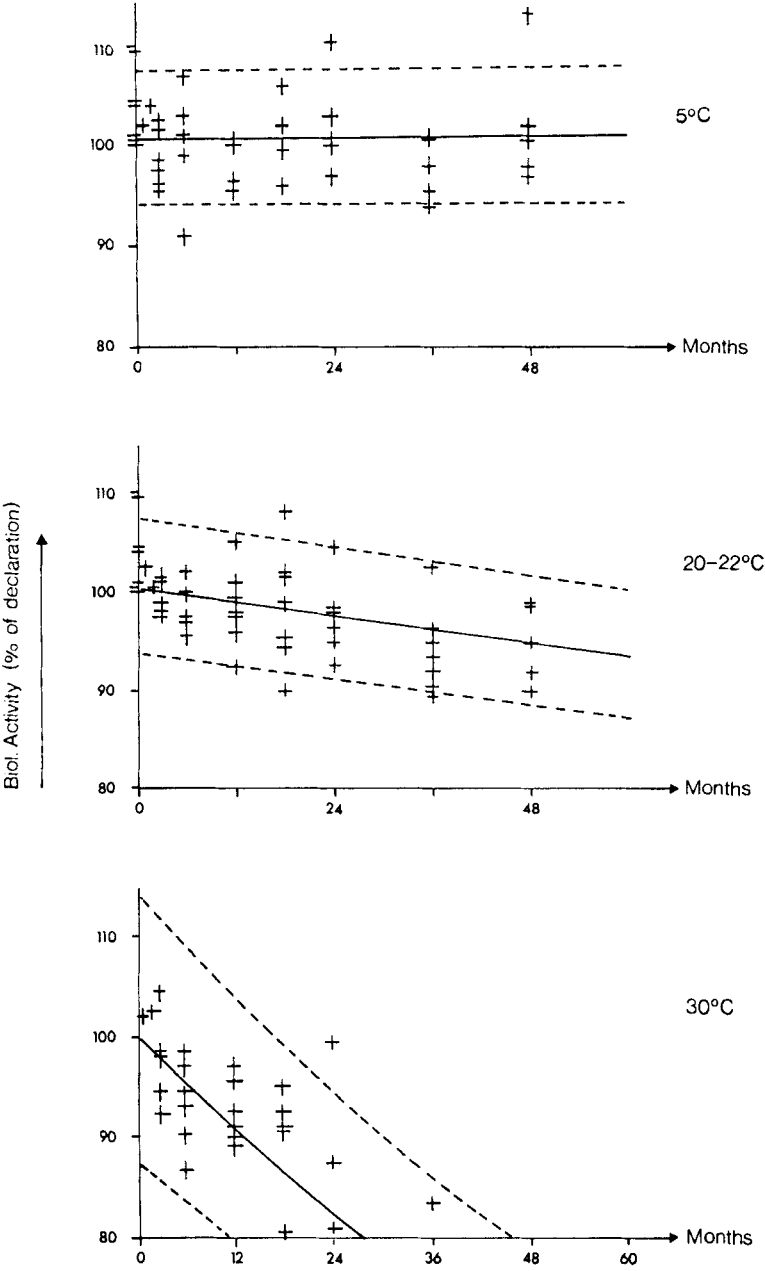


Figure 4.2 Shelf life of oxytocin concentrates

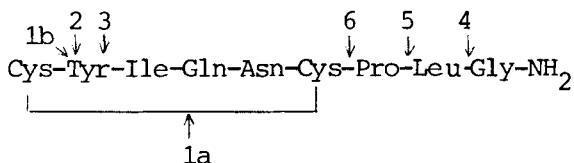
5. Metabolism

The natural concentration of oxytocin in human plasma is low. According to Chard et al. the level is less than 0.75 μ units/ml in healthy men and women (40), while Leake and Weitzmann published values of 1.4 - 1.7 μ units/ml (41). During labour there is a marked rise in oxytocin level: Kumaresan et al. found a concentration of 82 μ units/ml (42) and Leake and Weitzmann published a value of 6 μ units/ml (41).

The half-life of oxytocin in the blood is only a few minutes (43,44), the reason being that it is rapidly degraded mainly by the liver and kidneys. Two main enzyme systems are responsible for the inactivation of oxytocin. The removal of glycineamide from the C-terminal end of the oxytocin molecule has been demonstrated in all animal species investigated and in man, but the removal of Leu-Gly-NH₂ is confined to a few species (44,45,46). Furthermore, numerous other organs display peptidase activity and are able to split the oxytocin molecule. Extracts of rat brain inactivate oxytocin by un-specific peptidases (47) and similarly microsomal and soluble fractions of uterus and pancreas (48,49) and testis (50) inactivate oxytocin. Homogenised testis inactivates oxytocin by reduction of the disulphide bridge and cleavage of the ensuing cysteine-tyrosine peptide sequence.

Plasma-oxytocinase is formed in the uterus of pregnant women and released into the plasma. This aminopeptidase hydrolyses the hemicystinyl-tyrosine peptide bond to yield an acyclic compound (51).

The biological sites of inactivation of oxytocin according to Pliska and Rudinger (52) are shown in the following diagram:



1 a: SS-SH transhydrogenase

1 b: aminopeptidase, splits the molecule between the hemicystinyl-tyrosine residues

2 : serum oxytocinase

3 : tyrosinase

4 : carboxamidopeptidase

5 }
6 } : endopeptidases

6. Analysis

6.1 Identity tests

6.11 General tests

The methods of detection described in section 6.122 (thin-layer chromatography) which produce various colour reactions or fluorescence, may be regarded as general, unspecific identification tests for peptides.

6.12 Specific identification tests

6.121 Infrared spectrum

See section 2.1

6.122 Thin-layer chromatography

The R_f value and visualisation by various detection procedures is a specific criterion of identity.

A number of solvent systems, mainly based on butanol-acetic acid-water mixtures, are employed to develop silica gel thin-layer plates.

The solvent system butanol-acetic acid-water (4:1:1 parts by volume) is recommended in the literature for use with silica gel plates (53-55). According to Hase and Walter (54) who used Pauly reagent or chlorine-o-toluidine reagent to detect the spots oxytocin has an R_f value of 0.45.

Another butanol-acetic acid-water system (4:1:5) (upper phase) is stated by a number of authors (56-60) to be a suitable mobile phase.

Khan and Sivanandaiah (58), who used butanol - 0.1 N acetic acid-pyridine (5:11:3, upper phase) and silica gel G plates, reported an R_f value of 0.68 for oxytocin.

A solvent system comprising butanol-acetic acid-pyridine-water (15:3:10:12) for use with silica gel G plates has also been described (57,59,60). The last-mentioned author employed the ninhydrin or chlorine-o-toluidine reaction to visualise the spots.

Flouret *et al.* (61) worked with Eastman Chromagram silica gel thin-layer sheets, developing the chromatograms with methanol-chloroform-acetic acid-water (38:62:2:2) and using the Pauly and/or the chlorine-o-toluidine colour reaction to detect the spots ($R_f = 0.4$).

A solvent system derived from the above and modified as follows may be used to distinguish oxytocin from other nona-

peptides such as [8-lysine]vasopressin, [8-ornithine]vasopressin, [2-phenylalanine, 8-lysine]vasopressin, [des-1-amino]oxytocin (62).

Mobile phase: methanol-chloroform-acetic acid-water 30:70:1:6

Thin-layer plate: Commercial silica gel plates 60 F₂₅₄, MERCK, Darmstadt, Federal Republic of Germany, thickness 0.25 mm

Suitable methods to detect the spots are the Folin-Ciocalteu's reagent (MERCK), fluorescamine (63) and the usual ninhydrin and chlorine-o-toluidine reactions.

Samejima et al. (64) have developed a highly sensitive fluorescence spray reagent (phenylacetaldehyde-ninhydrin).

Nakamura and Pisano (65) have described TLC systems for separating several peptides, including oxytocin, derivatised with fluorescamine. The compound is dissolved in buffer solution, spotted on Merck commercial silica gel 60 plates and derivatised at the start line by development with or immersion in an acetone-hexane solution of fluorescamine. The R_f values were 0.46 with chloroform-isopropanol-water (2:8:1) and 0.89 with acetone-ethyl acetate-methanol-water (3:2:1:1) as the mobile phase.

6.123 Electrophoresis

Electrophoresis was carried out as described by Mühlemann et al. (53) in a moist chamber apparatus (CAMAG, Muttenz, Switzerland) using cellulose-coated plates with a thickness of 0.1 mm (MACHERY-NAGEL, Düren, Federal Republic of Germany) and applying a potential gradient of approximately 23 V/cm for 45-60 minutes. Pyridine-acetic acid-water 1:10:90, pH 3.6 was employed as the electrolyte. The mobility of oxytocin (relative to arginine) was reported to be m_{Arg}^* 0.29.

Flouret et al. (61) described the thin-layer electrophoresis of oxytocin on Eastman chromagram silica gel thin layer sheets in a Brinkmann-Desaga apparatus (400 V, 2 hours; using 0.1 N pyridine-acetic acid buffer pH 5.6).

6.124 Amino acid analysis

Detection of the amino acids after hydrolysis of the peptide may be regarded as an indirect method of identification. Oxytocin is normally hydrolysed with 6 N hydrochloric acid at 115°C for 16 hours in a sealed tube. The amino acids formed are separated by ion exchange chromatography, assayed after derivatisation either colorimetrically (ninhydrin) or fluorimetrically (Fluram^R, o-phthalaldehyde) and identified by comparing the retention times with those of a given amount of a test

mixture.

This method may be used to determine the amino acid ratio and the peptide content (see section 6.25).

6.125 Rat uterus method

- see section 6.31

Under the conditions described in the sections on assay methods in various pharmacopoeias, a solution of oxytocin induces contractions in uterine muscle.

6.2 Quantitative physico-chemical methods

6.21 Ultraviolet spectrometry

- see section 2.2

6.22 Fluorimetric methods

- see section 6.27

6.23 Colorimetric analysis

The peptide has been assayed using the well-known colour reactions, e.g. the ninhydrin reaction (66) and the Folin-Lowry reactions (67).

The method of Ellmann (68) has been used to determine the sulfhydryl content.

6.24 Determination of nitrogen (Kjehldahl)

The organic nitrogen of the peptide is converted to ammonium sulphate by the Kjehldahl method using concentrated sulphuric acid and a suitable catalyst. The solution is rendered alkaline and the ammonia is steam distilled into a receiver containing boric acid. The boric acid is then titrated potentiometrically with hydrochloric acid and the nitrogen, or the peptide content, calculated from the result.

6.25 Amino acid analysis

- see section 6.124

Amino acid analysis may be used to determine the amino acid ratio as well as the peptide content.

Analysis of oxytocin reveals the presence of aspartic acid, glutamic acid, proline, glycine, isoleucine and leucine in equimolecular proportions. Cystine and tyrosine must also be detectable, but since they undergo partial decomposition during hydrolysis, the quantity of these two amino acids is only about

70% of theory.

Further amino acids are not detectable in synthetic oxytocin. However, the presence of foreign amino acids, such as arginine, lysine, and phenylalanine, may serve as a criterion of purity for oxytocin of natural origin (see section 6.34). The peptide content is calculated from the yield of intact amino acids present.

6.26 Gel filtration

Various authors have described gel filtration methods using Sephadex G-15 and G-25 (54,56,57,61). This method which separates off dimers and polymers is mainly employed as a means of purification.

6.27 High performance liquid chromatography (HPLC)

Since the introduction of chemically modified silica gels as the stationary phase, HPLC on reversed phase C₈ or C₁₈ columns has come to the fore as the method of choice for the assay of oxytocin. Krummen and Frei estimated the oxytocin content of injections, tablets and oxytocin concentrate (69). Isocratic elution and short wavelength UV detection at 200-220 nm were adequate for the purpose. Figure 6.1 shows a typical chromatogram (70). Since the mobile phase (acetonitrile/phosphate buffer) readily transmits UV light at 200-220 nm, the lower limit of detection is approximately 30 ng (approx. 30 pmol) per injection (69). The HPLC results correlate very well with those obtained by bioassay. Figure 6.2 illustrates the good agreement between HPLC values and the results obtained in the rat uterus test for 38 different batches of oxytocin (70). The correlation coefficients and critical values are shown in Table 6.1.

Table 6.1 Correlation coefficients and critical values for the results in Figure 6.2, published by Krummen et al. (70).

Samples	Number of Data Pairs	Correlation Coefficient	Critical Value ⁺⁾
Liquid	25	0.9962	0.618
Solid	13	0.9995	0.801
Liquid + Solid	38	0.9969	0.513

⁺⁾ For n-2 degrees of freedom at the 0.1% level.

HPLC is so highly selective that it will also separate compounds closely related in structure. Figure 6.3 shows by way of example the separation of oxytocin and 3 stereoisomers having 1 or 2 amino acids with the D-configuration (70).

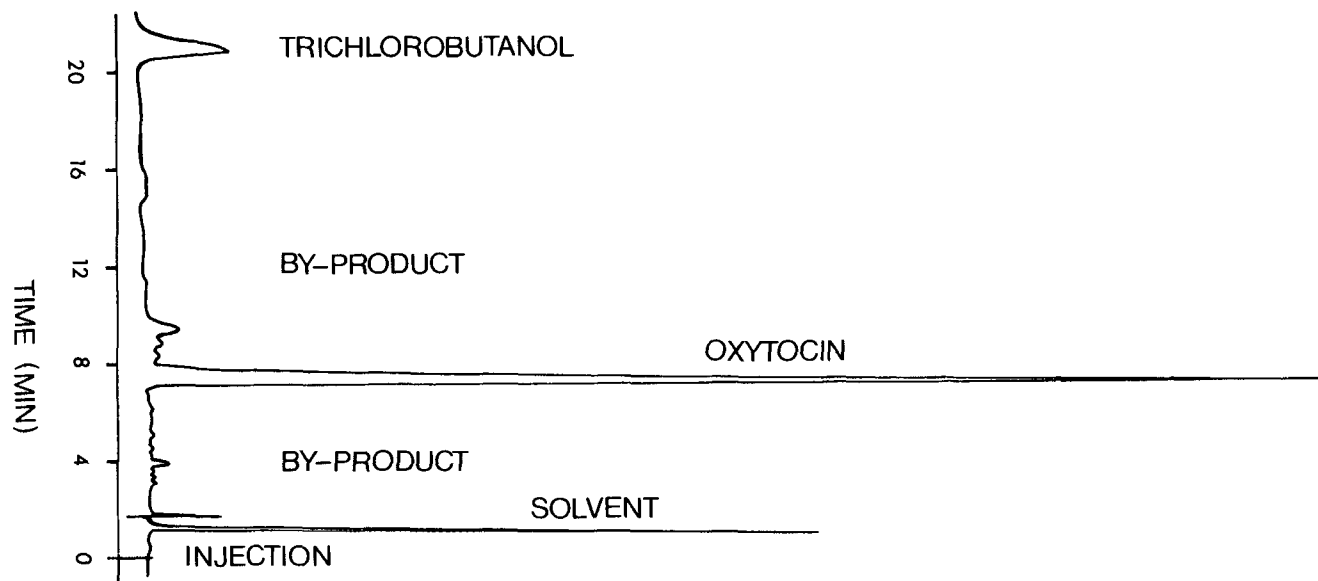


Figure 6.1 Chromatogram of 50 μ l of oxytocin concentrate (200 units/ml)

Conditions were as follows: Column RP 18, 10 μ m, 250 x 4.6 mm ID, isocratic elution with 18% acetonitrile in phosphate buffer solution (1/15 mol) pH 7, room temperature, flow rate 2.0 ml/min, pressure at column inlet 150 bars, UV monitor at 210 nm.

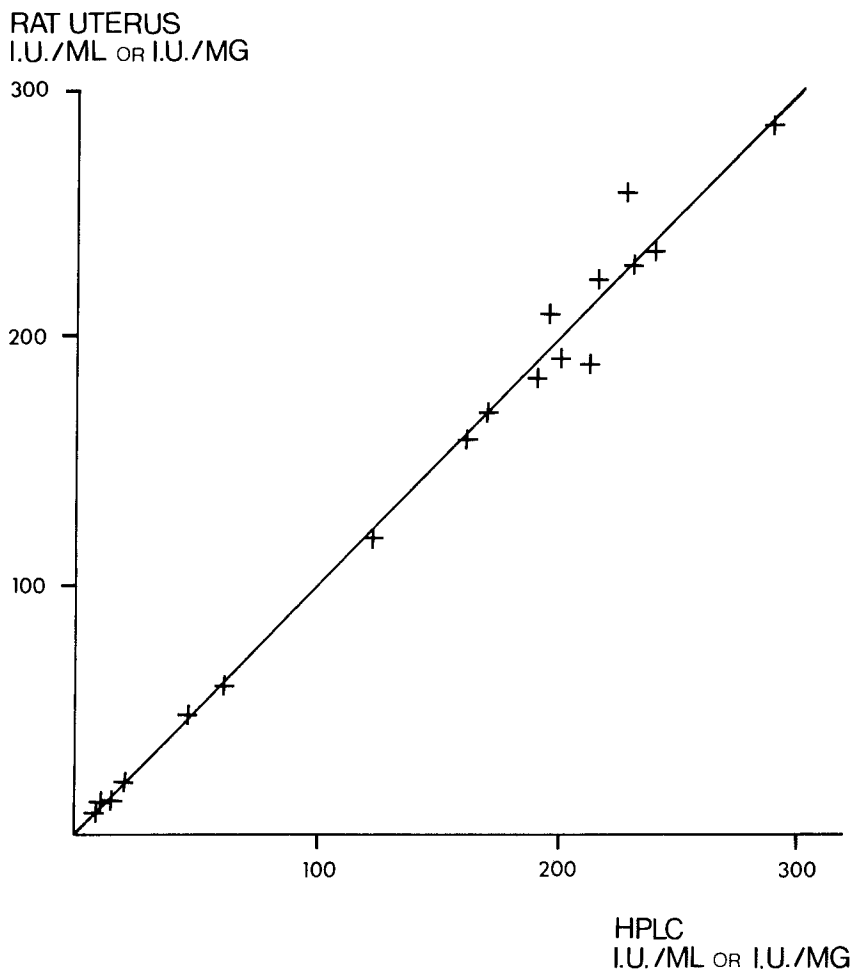


Figure 6.2

Correlation between the results of rat uterus and HPLC assays of oxytocin

Correlation coefficients and critical values for the results in Table 2.1

- 1 = Oxytocin
 2 = [D-Gln⁴] oxytocin
 3 = [D-Tyr², D-Gln⁴] -oxytocin
 4 = [D-Tyr²] oxytocin

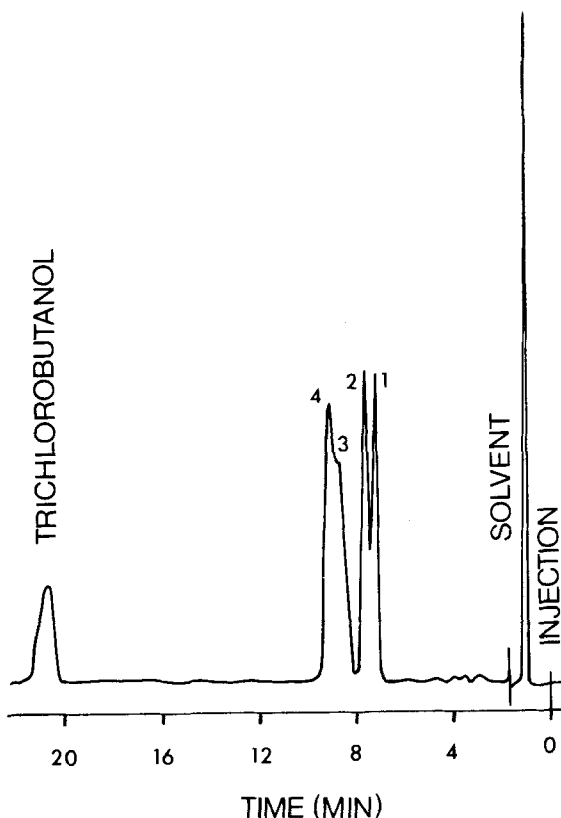


Figure 6.3

Chromatogram of 50 μ l of a mixture of oxytocin and diastereoisomers (= 100 μ g/ml each).

Conditions as for Figure 6.1

Larsen *et al.* (71,59) separated oxytocin from 7 of its stereoisomers (cf. Table 6.2) on a μ Bondapak C₁₈ column. Mixtures of 10% tetrahydrofuran or 18% acetonitrile or 16% dioxane in acetate buffer were used as the mobile phase. The nature of the organic solvent and the pH affect the separation. HPLC was found to be much superior to the classical separation on Sephadex G-25.

Table 6.2 The effect of the solvent on the separation of oxytocin and its diastereomers by reversed-phase HPLC; published by Larsen et al. (59).

Oxytocin diastereoisomers	10% THF 90%0.05M NH ₄ OAc pH 4.0 1.6 ml/min		18%MeCN 82%0.01M NH ₄ OAc pH 4.0 2.0 ml/min		16%Dioxane 84%0.05M NH ₄ OAc pH 4.0 1.5 ml/min	
	k'	α	k'	α	k'	α
oxytocin	7.73	1.00	7.31	1.00	7.70	1.00
4-D-Gln	9.12	1.18	8.33	1.14		
7-D-Pro	9.12	1.18	10.4	1.42		
6-Hemi-D-Cys	9.66	1.25	9.43	1.29		
5-D-Asn	11.1	1.44	9.28	1.27		
1-Hemi-D-Cys	13.1	1.69	14.6	2.00		
8-D-Leu	13.3	1.72	12.0	1.64	13.7	1.77
2-D-Tyr	17.7	2.29	10.7	1.46	12.7	1.67

Oxytocin may also be separated from other peptide hormones, e.g. [8-lysine] or [8-arginine] vasopressin by reversed-phase HPLC. The separation may be effected isocratically (72) or by gradient elution (73) with mixtures of aqueous buffers and acetonitrile, methanol, dioxane or tetrahydrofuran. The mobile phase must contain a minimum concentration of salt, since otherwise the separation efficiency is low. The chromatographic separation is influenced by the nature of the organic solvent and also by the pH and salt concentration, but these effects are smaller for oxytocin than for other similar peptides (69).

Nachtmann employed an isocratic method and short wavelength UV detection (74) to test the purity of intermediates used in the synthesis of oxytocin. The lower limit of detection was in the ng range both for free and for protected peptides. The primary amine group of the N-terminal, hemicycstine of oxytocin may be derivatised with FLURAM^R and assayed fluorimetrically. Gruber et al. (75) carried out the derivatisation in phosphate buffer at pH 7 before chromatographic separation on Partisil ODS with a linear gradient of 15 to 50% acetone in 0.03% ammonium formate and 0.01% thiodiglycol. 15 pmol of the oxytocin derivative gave an easily detectable peak with a signal to noise ratio of 15:1. This method was used by Live et al. to test the purity of synthetic oxytocin (76,60). It was possible in this way to separate oxytocin from 16 similar peptides.

Radhakrishnan et al. separated oxytocin from other polypeptides, such as [8-arginine] vasopressin, on Partisil SCX cation exchangers using volatile pyridine acetate buffers (77). An automated fluorescamine column monitoring system was used for detection.

Postcolumn derivatisation with FLURAM^R was employed by Frei *et al.* (78,79) and Krummen *et al.* (70). The lower limit of detection was 5 - 10 ng (5 - 10 pmol) per injection. The compound was chromatographed on reversed-phase C 8 or C 18. Since the sample solutions are concentrated in the column, very dilute solutions can still be determined with great precision.

6.3 Biological methods

The **activity** of a sample of oxytocin is determined by comparing it with the International Standard Preparation or with a preparation which has been standardised against the International Standard.

At the present time the standard preparation for determination of oxytocin activity is the Fourth International Standard for Oxytocin for Bioassay (80). The standard preparation for the determination of vasopressor activity is the First International Standard for Lysine Vasopressin (80). Both standards (highly purified peptides of synthetic origin) supersede the Third International Standard for Oxytocin and Vasopressin, Bovine, for Bioassay, which was an acetone-dried extract of posterior lobes.

Numerous biological assays have been known for many years and have been the subject of detailed reviews (81-87). For the experimental procedures the reader is referred to these papers and to the methods described in the various pharmacopoeias. Only methods included in the most important pharmacopoeias will be discussed here. Oxytocin as an active principle is not described in any of the pharmacopoeias. The stipulated contents as a percentage of the declared composition relate to oxytocin injections.

6.31 Rat uterus method

The **contractions** induced in isolated rat uterus by the oxytocin sample are compared with those induced by a standard preparation and evaluated as described in the pharmacopoeias.

Pharmacopoeia ⁺⁾	Stipulated potency of injections (Confidence limits, P = 0.95)	
B.P.		
Eur.P.	90 - 111%	(80 - 125%)
Swiss		
Nord.		

⁺⁾ Abbreviations of the pharmacopoeias as in Martindale, The Extra Pharmacopoeia, 27th Edition

6.32 Chicken blood pressure method

The depressor effects of the sample and standard on the chicken blood pressure are measured and evaluated in accordance with detailed instructions given in the following pharmacopoeias.

Pharmacopoeia ⁺)	Stipulated potency of injections (Confidence limits, $P = 0.95/L =$ confidence interval)	
B.P.		
Eur.P.	90 - 111%	(80 - 125%)
Swiss		
Nord.		
U.S.	85 - 120%	(L ≤ 0.20)
Jap.	85 - 120%	(L ≤ 0.15)

6.33 Milk ejection assay

This method is based on measurement of the milk ejection pressure in a lactating rat and is proposed in the 1978 addendum to the 1973 British Pharmacopoeia as an alternative to the other two methods (rat uterus and chicken blood pressure).

6.34 Rat blood pressure assay

This method measures the pressor effect of oxytocin samples. It sets a limit to the content of vasopressin which may occur as a impurity in oxytocin of natural origin. This test is not required when the oxytocin is prepared by synthesis and the absence of foreign amino acids is demonstrated by amino acid analysis (88,89).

However, synthetic oxytocin itself has a small intrinsic pressor effect in mammals (88,90,91). The synthetic highly purified oxytocin described in section 1.7, which was shown by HPLC to contain less than 1% of peptide impurities, had a vasopressor activity amounting to 0.9% and 1.09% of its oxytocin activity, according to the results obtained in two independent laboratories (92).

Below are shown requirements listed in the most important pharmacopoeias. The USP limit is inconsistent with the above-mentioned intrinsic vasopressor activity of pure oxytocin.

⁺) Abbreviations of the pharmacopoeias as in Martindale, The Extra Pharmacopoeia, 27th Edition

Pharmacopoeia +)	Stipulated maximum vasopressor activity as a percentage of oxytocic activity
B.P.	≤ 5%
Eur.P.	≤ 2.5%
Swiss	≤ 5%
Nord.	≤ 5%
U.S.	≤ 1%
Jap.	≤ 5%

6.4 Determination in biological material

6.41 Bioassays

The most commonly employed bioassays for oxytocin in biological fluids are based on the contractile effect on the myometrium or mammary myoepithelium in vitro or in vivo. Measurement of the depressor effect in the chicken is less sensitive. The various methods which differ in their selectivity, sensitivity and precision have been evaluated by Munsick as shown in Table 6.3 (87).

The details may be found in the numerous reviews (83, 87, 93, 94). The rat milk ejection test is the most sensitive method, the lower limit of detection being 2.5 - 5 μ units/ml (87, 94).

Despite the relatively high specificity of the bioassay methods, accompanying substances frequently interfere so that prior extraction procedures are necessary and these are usually time consuming and not particularly reproducible. Affinity chromatography on agarose-bound neurophysin may be a suitable alternative (see 6.42).

⁺) Abbreviations of the pharmacopoeias as in Martindale, The Extra Pharmacopoeia, 27th Edition

Table 6.3 A qualitative evaluation of several methods used to assay oxytocin (1 + least; 3 + most); published by Munsick (87)

	Speci- ficity to Oxytocin vs. Other Neuro- hypo- physial Peptides	Speci- ficity to Oxytocin vs. Other extra- neous sub- stances	Sensitivity to Oxytocin	Rapid Screen- ing Assay for Oxytocin	Precision of Assay	Expense of Equip- ment for Exper- imental Setup	Expe- rience Required	Set up Time per Assay Prepa- ration
Isolated rat uterus								
0 Mg ²⁺	3+	1+	1-2+	3+	2+	1-2+	1-2+	1-2+
0.5 mM Mg ²⁺	2+	1+	1-2+	3+	2+	1-2+	1-2+	1-2+
Rabbit, guinea pig or rat milk-ejection, iv	2+	2-3+	2+	2+	2-3+	3+	2+	2+
Rat or guinea pig milk-ejec- tion, i.a.	2+	2+	3+	1+	2+	3+	3+	3+
Avian vasode- pressor	2+	2-3+	1+	2+	2-3+	2+	1+	2+

6.42 Radioimmunoassays (RIA)

Owing to its low molecular weight oxytocin is not a good antigen and initially the production of antibodies with a high titre presented difficulties. Gilliland and Prout showed that antibodies could be produced by administering natural unconjugated oxytocin with Freund's adjuvant to rabbits (95). However, the antibody titre was low. Despite this handicap, a radioimmunoassay was developed using this technique, but it was no more sensitive than the best bioassay methods (96,97).

Better results were obtained with oxytocin adsorbed on carbon microparticles (40,98) and with oxytocin conjugated with bovine serum albumin (99). The latter method has been taken up by several authors (100,101). Rabbits are used to produce the antibodies. Nevertheless, only in a few laboratories has it proved possible to prepare usable antibodies (102), and this has so far prevented the application of RIA for oxytocin on a wider scale (103). Now, however, oxytocin antiserum is available commercially (104). The hormone labelling is usually accomplished by iodination with ^{125}I using the chloramine-T method (103). Dawood *et al.* labelled oxytocin by the lactoperoxidase method (105).

For details of RIA methodology, the reader is referred to the original papers cited and to the various reviews (88, 93,103,106).

Problems are also encountered in extracting oxytocin from blood serum. Chard *et al.* have published a method in which oxytocin is adsorbed from plasma on Fuller's earth and eluted with aqueous acetone (40). However, recovery is not particularly good (50 - 60%) and the reproducibility leaves something to be desired (101). Some authors therefore omit the extraction step and use blood serum directly for the RIA (100,104).

An extract based on affinity chromatography on agarose-bound neurophysin has recently been described (107). Oxytocin can be extracted from plasma, urine and cerebrospinal fluid with high recovery and high specificity by this method.

The lower limit of detection of RIA has been variously reported as 2.0 $\mu\text{units/ml}$ (104) and 0.05 $\mu\text{units/ml}$ (103).

6.43 High performance liquid chromatography (HPLC)

Gruber *et al.* (75) determined the oxytocin content of rat posterior pituitaries using the method described in 6.27. These authors point out that the method can be improved by using a

new fluorescence reagent.

HPLC may be expected to play an important part in future in the assay of oxytocin in biological material.

6.5 Determination in dosage forms

Before HPLC became available, oxytocin in pharmaceutical dosage forms was usually assayed by the chicken blood pressure method or rat uterus method, which were adopted by most pharmacopoeias (cf 6.3).

HPLC has recently been developed and is now the method of choice. It is simpler as regards the apparatus needed, cheaper and more rapid than the bioassays and it is appreciably more accurate (70,108). It is also sufficiently specific to separate oxytocin from byproducts of synthesis (e.g. stereoisomers of the active compound) or related peptides (e.g. 8-lysine vasopressin) (cf. 6.27). This method may also be used for stability tests on the various oxytocin dosage forms (69,70).

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PENICILLAMINE

Ching Ching Chiu and Lee T. Grady

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1. Description

Penicillamine is the characteristic degradation product of penicillin type antibiotics. It was first isolated and characterized by Abraham and co-workers in 1943 (1). It is used medically as treatment of medical problems such as Wilson's disease (too much copper in the body), rheumatoid arthritis, cystinuria, and lead poisoning (2).

1.1. Nomenclature

1.1.1. Chemical Names:

D-3,3-dimethylcysteine, D-3-mercaptovaline, β -mercaptovaline, β,β -dimethylcysteine, D- β -thiovaline, α -amino- β -methyl- β -mercaptobutyric acid, and D-valine,3-mercapto.

1.1.2. Generic Name:

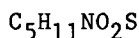
Penicillamine is the United States Adopted Name (3) for D-penicillamine.

1.1.3. Trade Names:

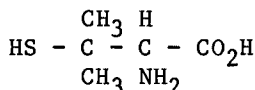
Cuprimine (4) (Merck-Sharp and Dohme), Cuprenil (4), Depamine (4), Trolovol (4), DMC (4), Depen (3) and Distamine (4) (Wallace).

1.2. Formula

1.2.1. Empirical



1.2.2. Structural



1.3. Molecular Weight

MW = 149.21

1.4. Appearance, Color, Odor, Taste

Penicillamine is a fine white or practically white

crystalline powder having a slight characteristic odor and a slightly bitter taste.

2. Physical Properties

2.1. Crystal Properties

2.1.1. Polymorphism

Evidence for the existence of polymorphism for D-penicillamine is from infrared spectroscopy and X-ray crystallography. It was reported that polymorph I has a minimum between peaks at 1078 and 1101 cm^{-1} that are less intense than the adjacent peaks at about 1050 and 1160 cm^{-1} , while polymorph II has an absorption peak at 1092 cm^{-1} which is more intense than the adjacent peaks at about 1050 and 1160 cm^{-1} (5).

Under the polarizing microscope (6), both polymorphs were seen as anisotropic crystals, I as needles and II as plates. X-ray crystallography also confirmed the occurrence of two polymorphs of D-penicillamine (6).

2.1.2. X-Ray Crystallography

X-ray crystallographic data on D-penicillamine.HCl.H₂O were reported by Crowfoot (7). The unit cell characteristics listed for D-penicillamine.HCl.H₂O are: $a = 6.85$, $b = 6.08$, $c = 12.20$, $\beta = 103.6^\circ$, $\rho = 1.360$; space group = $P2_1$, $N = 2$. Morphology: laths {101} elongated along {010}.

X-ray crystallographic data on L-penicillamine.HCl.H₂O also were reported (8).

2.2. Infrared spectrum

The infrared spectrum of D-penicillamine as Nujol mull was reported (9).

The infrared spectrum of DL-penicillamine was also described. The spectrum was taken as Nujol mull on cesium iodide windows and recorded on a Perkin-Elmer 180 Spectrophotometer (10).

The infrared spectrum of USP Reference Standard Penicillamine (Lot G) obtained as Nujol mull, using a

Beckman IR 4250 is shown in Figure 1. Table I lists the characteristic frequencies of penicillamine.

Table I
IR Spectral Assignment for Penicillamine

Frequency (cm^{-1})	Assignment
3170	$^+\text{-NH}_3$
2920	$^+\text{-NH}_3$
2600	-SH
1615	-CO_2^-
1280	-CO_2^-

2.3. Raman Spectrum

The Raman spectrum of DL-penicillamine was reported (10). It was obtained for powders by use of a modified rotating cell on a Jarrell-Ash 25-100 Spectrometer equipped with argon-ion (5145 Å) excitation. Some characteristic Raman bands for DL-penicillamine are listed in Table II.

2.4. Nuclear Magnetic Resonance Spectra

Proton magnetic resonance and ^{13}C magnetic resonance spectra were reported for DL-penicillamine (10). Proton and ^{13}C nuclear magnetic resonance spectra for penicillamine (Figures 2 and 3) were obtained on a Varian FT-80A NMR Spectrometer. The sample (about 200 mg) was dissolved in 3 ml of D_2O with DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) added as an internal standard. The proton and ^{13}C chemical shifts obtained for D-penicillamine are summarized in Tables III and IV, respectively.

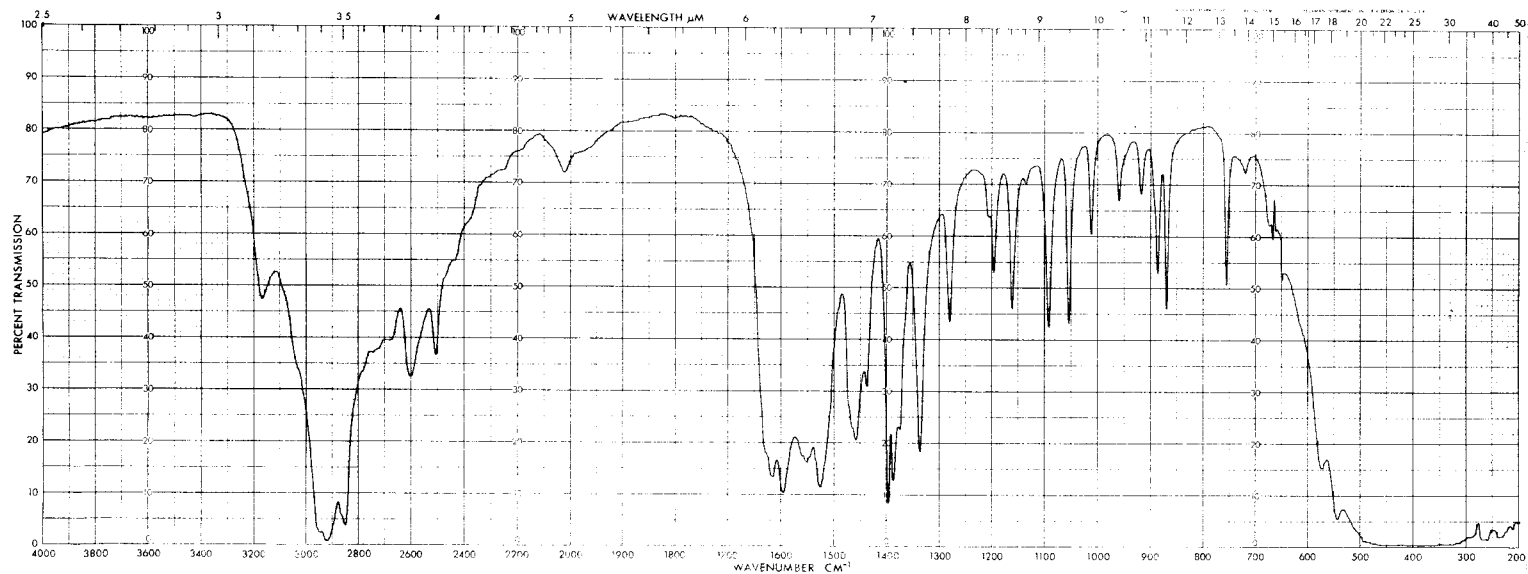


Figure 1. Infrared Spectrum (Nujol mull) of Penicillamine.
Instrument: Beckman IR 4250

Table IICharacteristic Raman Bands for DL-Penicillamine

Band Frequency (cm^{-1})	Characteristics	Assignment
2569	vs*	-SH
1659	w,* br*	-NH_3^+
1510	w,* br*	-NH_3^+
1597	w,* br*	-CO_2^-
1399	m* w*	-CO_2^-
576	s*	-NH_3^+
552	s*	-CH_2^-

*vs = very sharp, w = weak, br = broad, mw = medium weak

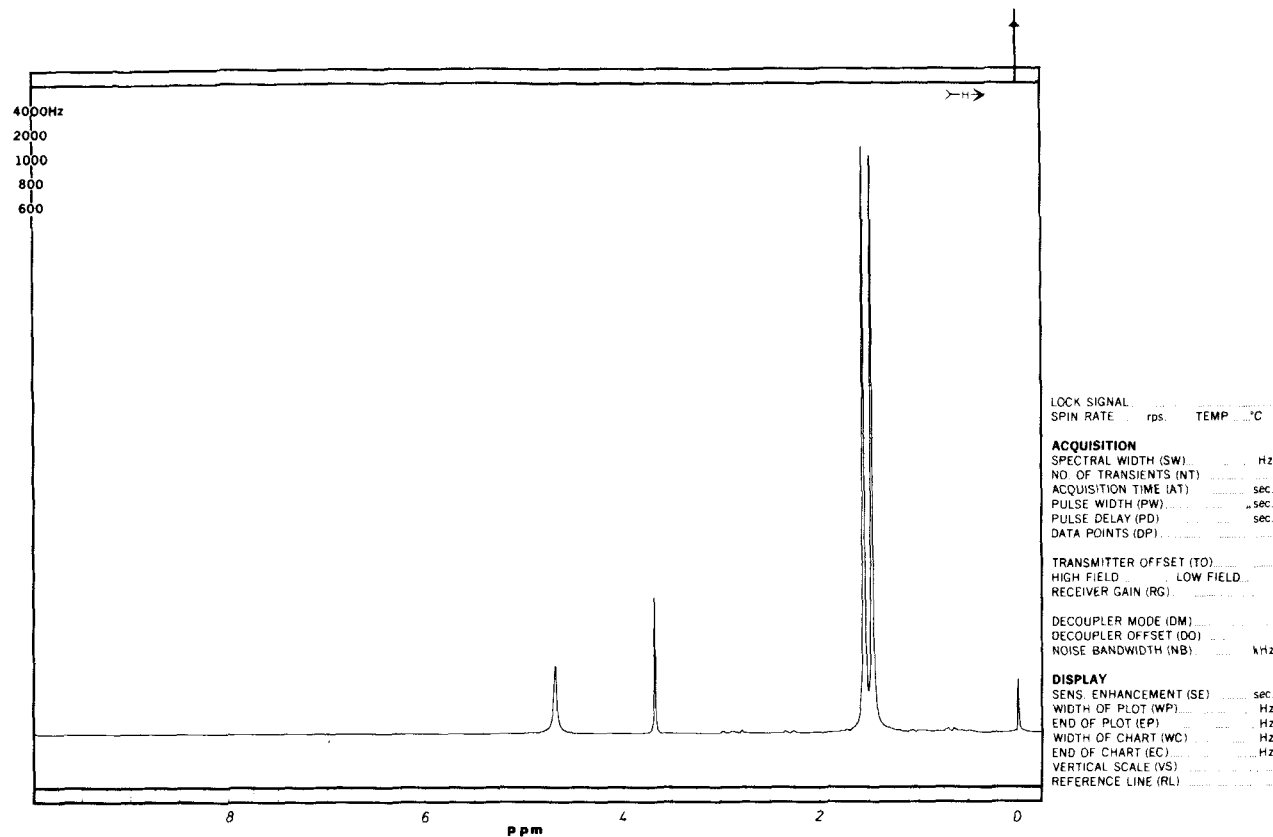


Figure 2. ^1H Nuclear Magnetic Resonance Spectrum of Penicillamine.

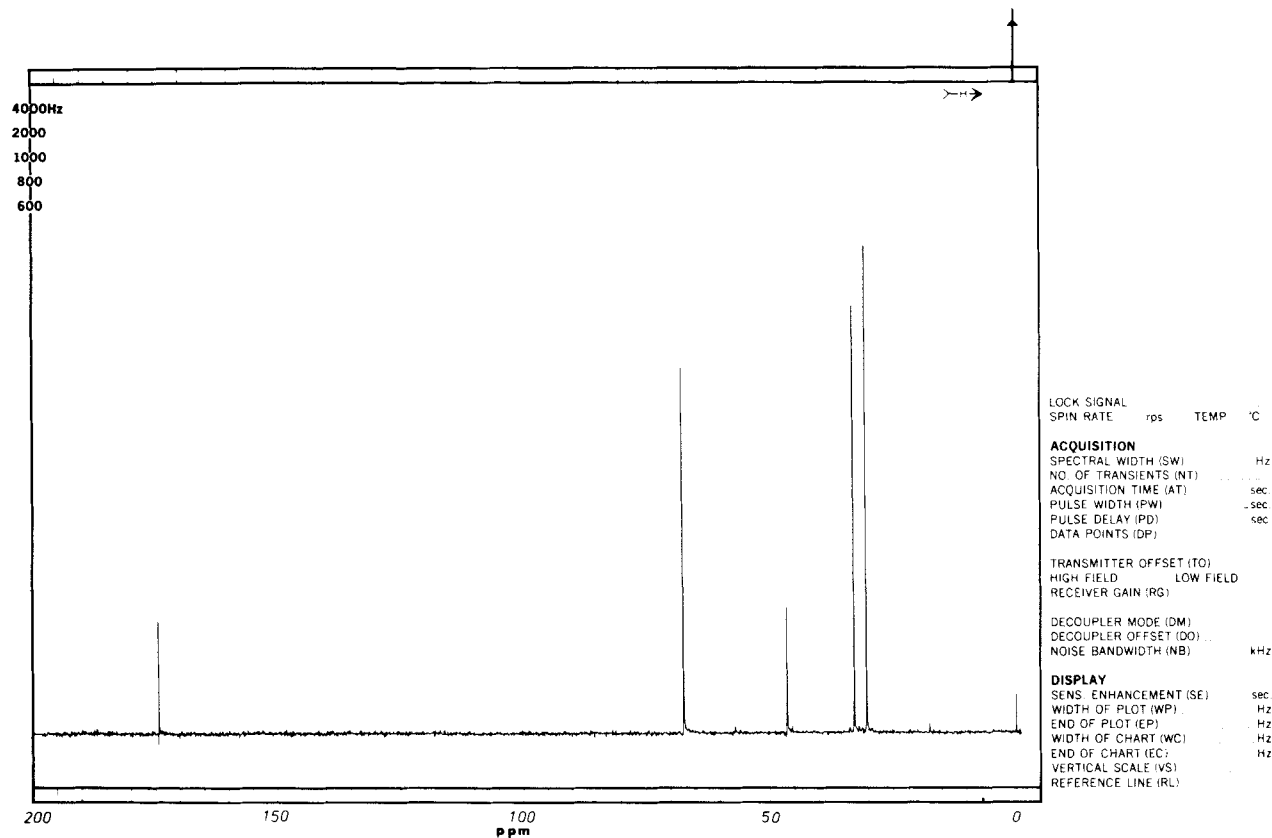
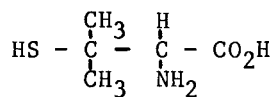
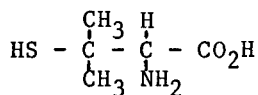


Figure 3. ^{13}C Nuclear Magnetic Resonance Spectrum
of Penicillamine

Table III¹H NMR Spectral Assignments for Penicillamine

Proton	Chemical Shift (ppm) (relative to -CH ₃ of DDS)	Multiplicity
-CH ₃	1.48, 1.56	singlet
-CH	3.69	singlet

*4.71 ppm from H₂O

Table IV¹³C NMR Spectral Assignments for Penicillamine

Carbon	Chemical Shift (ppm) (relative to -CH ₃ of DSS)
H-S-C-(CH ₃) ₂	32.90, 30.39
H ₂ N-CH-CO ₂ H	67.28
HS-C-(CH ₃) ₂	46.46
-CO ₂ H	173.94

2.5. Mass Spectra

Ultramicrodetermination and selective identification of penicillamine have been achieved by the combined gas chromatography-mass spectrometry technique (11-13). Penicillamine was derivatized with N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA)-1% trimethylchlorosilane in pyridine (11) or N-trifluoroacetamide-L-prolyl chloride (12). The derivatives were analyzed by gas chromatography. Two glass columns, 50 cm x 3 mm i.d. packed with 1.5% OV-101 on 60-80 mesh Diatoport S and 1 m x 3 mm i.d. packed with modified OV-101 on 100-120 mesh Chromosorb W (AW) were employed. Column temperature was programmed from 140° to 170° at the rate of 5° per minute. A Hitachi RMU-6MG mass spectrometer combined with an 002 Datalizer using Hitac-10 computer for an on-line data processing was used. Ionization energy and accelerating voltage were 70 eV and 3000 V, respectively. The ion source temperature was 210°. The limit of detection of penicillamine was about 10⁻¹⁰ g level. The mass spectra of the fragment ions of N-TFA-L-prolyl-n-butyl ester and N-TMS-O-TMS ester of penicillamine are summarized in Tables V (11) and VI (12), respectively.

Table V

Fragment Ions of N-Trimethylsilyl-O-trimethylsilyl Ester
Derivative of Penicillamine

m/e	Relative Intensity
147	21.4
128	3.8
100	5.0
75	9.4
74	8.4
73	100.0
59	6.4
45	17.7

Table VI
Fragment Ions of N-TFA-L-Prolyl-n-Butyl
Ester of Penicillamine

m/e	Relative Intensity
324	17.0
250	42.4
167	24.0
166	17.8
73	13.3
70	15.9
55	15.0
41	33.0
29	35.5
28	29.5

2.6. Optical Rotation

The reported optical rotation values for D-penicillamine are:

$$[\alpha]_{\text{D}}^{25^{\circ}} = -63^{\circ} \quad C = 0.1 \text{ in pyridine (4)}$$

$$[\alpha]_{\text{D}}^{26^{\circ}} = -61.3^{\circ} \quad C = 2.5 \text{ in } 1.0 \text{ N NaOH (9)}$$

The specific rotation range required by official compendia (14, 15) is between -58° and -68° determined in a 5% solution in 1.0 N sodium hydroxide at 25°C .

2.7. Melting Range

The melting point temperatures reported for penicillamine are:

$$\begin{aligned} \text{m.p.} &= 198^{\circ}\text{C (1)} \\ \text{m.p.} &= 198.5^{\circ}\text{C (4)} \\ \text{m.p.} &= 212^{\circ}\text{C (9)} \end{aligned}$$

2.8. Differential Thermal Analysis

The only thermal event in the differential thermal analysis curve of D-penicillamine is the melting endotherm at 185° (6). Either polymorph of D-penicillamine gives same endotherm.

2.9 Solubility

Penicillamine is soluble in 9 parts of water, soluble in 530 parts of ethanol (95%), slightly soluble in other alcohols, practically insoluble in chloroform and ether at 20°C (14).

2.10. Acid-Base Properties

A titration curve of penicillamine hydrochloride at 25°C was first presented by Abraham (1),(16). The curve revealed the presence of three ionizable groups with pKa values of 1.8, 7.9, and 10.5, which correspond to the carboxyl, α -amino and β -thiol group.

Recently, the ionization constants for acid functions of D-penicillamine were verified by pH titration at 37°C and 0.15 M ionic strength (17, 18). These results correspond to that previously obtained by other workers (19-23).

2.11. Polarography

The polarographic behavior of penicillamine was studied by differential pulse polarography (24). The anodic polarographic wave was shown to be diffusion-controlled. A rectilinear calibration plot was obtained in citrate-phosphate buffer at pH 2.5 over the range $0.1-5.0 \times 10^{-6}$ M. The peak potential varied rectilinearly over the pH range 2-8 [$E_p(V) = -0.030-0.059$ pH]. The peak current is highly dependent on pH. It has maximum values at pH 3 and 10 (4.7 and 3.8 μA respectively) with a minimum value (3.2 μA) at pH 7.

3. Preparation

It is prepared by acid hydrolysis of penicillin (16) followed by precipitation from the hydrolysis mixture as the mercuric salt which is then collected, suspended in water and treated with hydrogen sulfide to liberate the free acid. Purification involves only recrystallization from water (16),(25). Several modifications of the preparation

procedure have been made to either increase the yield or simplify the process (26-28).

4. Synthesis

Penicillamine was first synthesized independently by several groups utilizing a series of reactions involving benzyl mercaptan and 2-phenyl-4-isopropylidene oxazolone (29, 30), (Figure 4). This involves the addition of benzyl mercaptan to 4-isopropylidene-2-phenyl-5(4)-oxazolone (I) using sodium methoxide as catalyst. Mild hydrolysis of the addition compound (II) gives (III), from which the benzoyl group was removed by strong acid hydrolysis. The S-benzyl amino acid (IV) was reduced with sodium and liquid ammonia to give the racemic penicillamine hydrochloride (V). Alternately, IV is first resolved by crystallization of the brucine salts of the N-formyl derivatives (VI) into the components D- and L-S-benzyl penicillamines which in turn were reduced to the corresponding D- and L-penicillamines.

Several other synthetic routes for the synthesis of penicillamine were also reported (31).

5. Stability-Degradation

Penicillamine is relatively stable in both light and air. Aqueous solutions of D-penicillamine are comparatively stable at pH 2-4 (14). In aqueous solution, penicillamine degrades slowly by first order or pseudo-first order kinetics. A 3% solution of penicillamine hydrochloride stored under nitrogen in a sealed container at 20° decomposed to the extent of about 10% per year (32).

6. Chemical Reactions

1. On treating penicillamine with bromine water, the thiol group is oxidized to a sulfonic acid group and a crystalline compound called penicillaminic acid is obtained (33).

2. The nitrogen of the α -amino group of penicillamine reacts as α -amino nitrogen in the Van-Slyke procedure (33).

3. Penicillamine reduces ammoniacal silver nitrate (25).

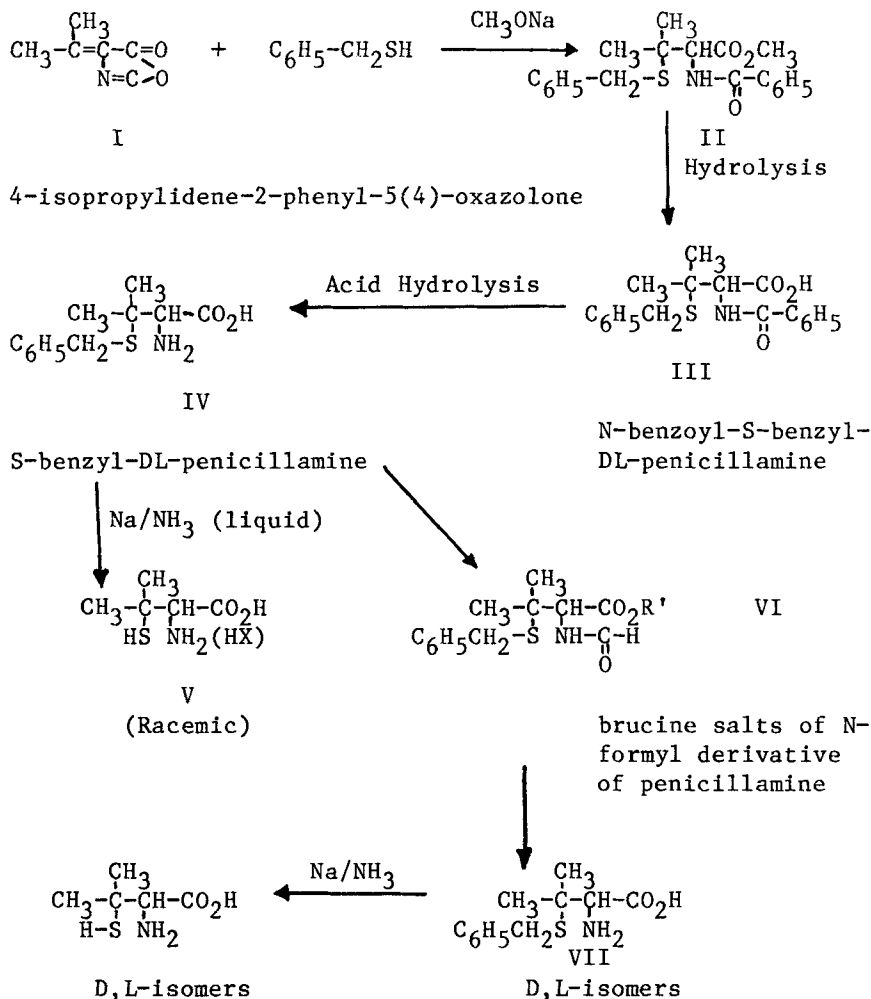


Figure 4. Synthesis of Penicillamine

4. On heating a solution of penicillamine and p-nitrophenylhydrazine in 1 N hydrochloric acid at 100°, glyoxal-p-nitro-phenylosazone is produced (25).

5. The kinetics and mechanism of the redox reaction of penicillamine in solution by chromium (VI) were investigated by stopped-flow technique. Three moles of penicillamine are required to reduce chromium (VI) to chromium (III). Several chromium (III) products have been identified by ion-exchange technique (34, 35).

7. Radiolysis

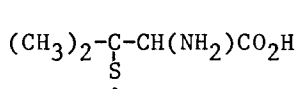
Radiolysis of penicillamine has been studied extensively using electron spin resonance spectroscopy at room temperature at 77°K (36, 37) and more recently at 4.2°K by Box (38, 39). These experiments sought insights into the application of penicillamine as radiation protective agent.

Solid state studies on crystalline penicillamine at 4.2°K have shown that electron addition to the carboxyl group is the major electron-capture route. At higher temperatures, dissociative electron capture to give NH_3 has also been observed (38,39).

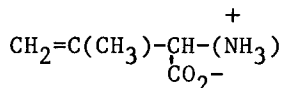
The effects of radiation dose and exposure time on the number of detectable paramagnetic resonance centers were investigated (40).

Recently penicillamine has been exposed to ^{60}Co gamma rays at 77°K, both in pure state and in dilute methanolic solution or aqueous glass (41). In methanol, the major step seems to be loss of the thiol group (.SH) to give the radical $\text{Me}_2\dot{\text{C}}\text{-CH}(\text{NH}_2)\text{CO}_2^-$ which is readily characterized by its esr spectrum. Loss of thiol (.SH) clearly dominates in the protic media.

Mechanistic aspects of the radiolysis of penicillamine in N_2O saturated solution at pH 5 was investigated (42). Penicillamine disulfide, penicillamine trisulfide and valine were formed. The formation of the latter two products was attributed to secondary reaction of radical I. Other minor products like ammonia and compound II were also found. These radiolysis results are in agreement with earlier studies (43-46).



I



II

Frozen aqueous mixtures containing a macromolecule and penicillamine (Sephadex or Thiogel and the reduced or oxidized form of penicillamine) were irradiated in vacuo with X-ray. In frozen aqueous mixture, radiation-induced unpaired spin may be transferred from a macromolecule to the penicillamine, whereas the intermolecular transfer of spins between solutes appeared to occur primarily during heat treatment. Electron spin resonance measurements were made at 77°K after heat treatment at 223°K (47).

8. Methods of Analysis

8.1. Elemental Analysis

The elemental composition of penicillamine is:

<u>Element</u>	<u>% Theoretical</u>
C	40.25%
H	7.43%
N	21.45%
O	9.39%
S	21.49%

8.2. Identification Tests

The following identification tests are listed in the compendia:

1. Addition of phosphotungstic acid solution to a penicillamine solution gives a deep blue coloration (14,15).

2. Addition of acetone-hydrochloric acid solution to a penicillamine solution gives a white precipitate (14,15).

3. Addition of ninhydrin test solution to a penicillamine solution gives a blue or blue-violet coloration (14,15).

4. Comparison of the infrared spectrum of the penicillamine sample with that of a reference standard (15).

8.3. Color Reactions

1. Penicillamine gives a deep blue coloration with ferric chloride (25).

2. Penicillamine gives a green color with Fehling's solution (25).

3. Penicillamine gives a strong purple coloration with alkaline sodium nitroprusside (33).

8.4. Colorimetric Analysis

Colorimetric methods of analysis were reported for penicillamine. These were based on the measurement of the absorption maximum at 645 nm of the blue coloration formed when penicillamine is reacted with tetrazolium salts in low concentration (48) (after 1 hr.) or with ferric chloride-potassium cyanide (49). (after 5 min.) at 65°C.

Quantitative determination of penicillamine during production process was based on orange color produced by penicillamine with Nessler reagent (50). The sensitivity is 3×10^{-6} g of penicillamine.

Colorimetric analysis based on copper (II) complex formation was also used (51). The absorption maximum is measured at 522 nm (after 30 min.). The method is selective for the unaltered drug in partially degraded solution.

Recently, a simple colorimetric method based on the use of Ellman's reagent, 5,5'-dithiobis-2(nitro-benzoic acid) was used for plasma penicillamine concentration (52).

8.5. Chromatographic Methods

8.5.1. Paper Chromatography

Table VII gives various paper chromatographic

systems used for the detection of penicillamine (53, 54). Ninhydrin spray reagent¹ has been used.

Table VII

Paper Chromatographic Systems for Penicillamine Analysis

Developing Solvent	Rf x 100	Reference
1-butanol-glacial acetic acid-water (120:30:50)	19	(52, 53)
phenol-water (160 g:40 ml) (solvent A)	50	(52, 53)
solvent A-conc. ammonium hydroxide (200:1)	55	(52, 53)
solvent A-ethanol-water (150:40:10)	33	(52, 53)
ethanol-water-conc. ammonium hydroxide (180:10:10)	11	(52, 53)
2-methyl-2-propanol-water-methyl ethyl ketone-diethylamine (80:80:40:8)	22	(52, 53)
methanol-water-pyridine (160:40:8)	42	(52, 53)
1-butanol-pyridine-water (60:60:60)	25	(52, 53)
pyridine-acetone-ammonia-water (45:30:5:20)	81	(54)
2-propanol-formic acid-water (75:12.5:12.5)	73	(54)

8.5.2. Thin-Layer Chromatography

Thin-layer chromatography was used mainly to detect the presence of penicillamine as impurity in penicillin type antibiotics or as conversion product from these

antibiotics. Table VIII summarizes the thin-layer chromatography systems for penicillamine analysis.

8.5.3. Gas Chromatography

Penicillamine is a polar nonvolatile molecule, derivatization is a requirement for analysis by gas chromatography. Jellum (60) reported the separation of amino thiols and diamino disulfides by reacting with pivalaldehyde at room temperature and neutral pH to form volatile neopentylidene derivatives which can be separated on a column of 5% SE-30 (on Aeropack 30) (5 ft x 1/8 in.) with a helium carrier-gas flow of 35 ml per minute. The temperature was programmed from 80° to 250° at a rate of 10° per min. The structures of the derivatives were confirmed by mass spectrometry.

Recently, penicillamine was analyzed as a cyclic oxazolidine derivative (61) by reacting with formaldehyde followed by 1,3-dichlorotetrafluoroacetone. The separation was done on a 3% OV-17 or SP-2250 (2 m x 2 mm) on 80-100 mesh support or 3% SE-30 (1 m x 2 mm) on 45-60 mesh Chromosorb WAW DMCS column using a flame ionization detector. The nitrogen flow rate was 30 ml per minute. Detector and injector temperatures were 250 and 200°C respectively. Column temperature was linearly programmed from 120 to 240° for both columns, at 8°C per minute. Glass as the column material and silanization of the chromatographic support are basic requirements, without which the analysis fails.

8.5.4. Pressurized Liquid Chromatography

Kenyhercz (62) developed an assay for penicillamine involving pressurized liquid chromatography with an electrochemical detector. A 50 cm x 2 mm glass column dry packed with a pellicular cation-exchange resin and aqueous mobile phase consisting of 0.018 M H₂SO₄ and either 0.1 M or 0.2 M Na₂SO₄ were used.

Table VIII

Thin-Layer Chromatographic Systems for Penicillamine Analysis

Stationary Phase	Developing Solvent	Visualization	Reference
Cellulose powder MN-300	2-propanol-butanone-1 <u>N</u> HCl (60:15:25)	Ninhydrin-cadmium acetate reagent	(55)
	2-methyl propanol-2-butanone- propanone-methanol-water- conc. ammonia (40:20:20:1:14:5)		(55)
Silica Gel G	butyl acetate - methanol - butanol - H ₂ O - acetic acid (75:45:45:30:7.5)	2% ninhydrin in ethanol (w/v)	(14)
Silica Gel	2-isopropanol - H ₂ O (85:15) 1-butanol - acetic acid - H ₂ O (72:18:18) Methanol - H ₂ O (64:36)	Ninhydrin spray	(57)

Table VIII - Cont'd.

Stationary Phase	Developing Solvent	Visualization	Reference
Silica Gel G	1-butanol - H ₂ O - ethanol - acetic acid (5:2:1.5:1.5)	(1) Ninhydrin spray (2) Spray consecutive- ly with the follow- ing reagents	(58)
	1-butanol - H ₂ O - acetic acid (4:1:1)	(a) 2 N NaOH	
	acetone - acetic acid (95:5)	(b) Iodine-azide	
	85% aqueous acetone	(c) 1% starch solution	
Silica Gel G/F	barbital acetate buffer - acetone (94:6)	Bioassay with Sarcina Lutea (ATCC 9431)	(59)

Recently, a method for the determination of the reduced and total penicillamines in biological fluids was reported (63). The separations were performed on a 30 cm x 0.2 cm column of Zipax SCX strong cation-exchange resin with a 3 cm x 0.2 cm precolumn of Zipax SCX. A pH 3.0 phosphate-citrate buffer of 0.04 M ionic strength, deaerated with oxygen-scrubbed nitrogen, was used as eluent with a flow rate of 0.6 ml per minute. Separations were detected with a mercury-based electrochemical detector. Electrochemical detectors were also applied in monitoring the separations of penicillamine (64, 65).

Blaha (66) reported the analysis of penicillamine as degradation product of penicillin G potassium by HPLC. The liquid chromatograph used was equipped with a UV detector operating at 254 nm and a column packed with Bondapak AX/Corasil (0.61 m x 2.3 mm). The mobile phase was a 0.1 M citric acid-0.2 M disodium phosphate buffer solution eluting at a flow rate of 0.7 ml per minute. Penicillamine was eluted with a retention time of 4.5 min.

Recently, a simple method for the determination of penicillamine in serum at therapeutic levels was described (67), 50-300 mg of penicillamine can be determined.

The analysis was based on a fluorescence derivatization of the sulfhydryl group with 5-dimethylaminonaphthalene-1-sulfonylaziridine combined with pressurized liquid chromatographic separation and fluorescence detection. Separation was achieved with a LiChrosorb RP-18 column (25 cm x 4.6 mm) with a mobile phase consisting of acetonitrile- (pH 8.2, 0.0033 M) phosphate buffer (1:2) + 0.05% ethylenediamine maintained at a flow rate of 1 ml per minute. The retention time for the derivatized penicillamine was 8 minutes.

Chromatographic separation of penicillamine and other sulfur containing amino acids (68) was performed on 60 cm x 0.6 cm column of Amberlite CG-4B anion-exchange resin (in the chloride form) operated at room temperature and 200 psi pressure. The column was eluted with 0.1 M HCl and maintained at a flow rate of 1.0 ml per minute. Iodoplatinate reagent (chloroplatinic acid-potassium iodide) was used for detection.

8.5.5. Ion-Exchange Chromatography

Penicillamine present in urine and plasma was

determined by conversion to penicillaminic acid and then chromatographed on a Dowex 1-X 8 column. The mobile phase consisted of 0.25 M formic acid, 0.2 M NaOH plus 5 ml of Brij 35 per liter (pH 4.2). The effluent was treated with ninhydrin (69).

Earlier, Frimpter (70, 71) reported the separation of penicillamine with sulfonated polystyrene cation-exchange resin column.

A column of 16 mm diameter containing 18 g of polyamide covered with 3.6 ml of tributyltin chloride was used to separate penicillamine. Tributyltin chloride has the properties of a liquid anion exchanger possessing special affinity for mercapto groups (72, 73). The column was eluted with buffer solutions of pH decreasing from 8 to 4 with gradient elution by addition of solvent A (0.1 M citric acid + 0.2 M boric acid + 0.2 M monosodium phosphate) to 50 ml of a solution of four parts solvent B (0.3 M disodium phosphate + 0.1 M trisodium phosphate + 0.5 g disodium EDTA) and one part solvent A.

8.6. Automated Analysis

Automation for analysis of penicillamine has been described. Chromatographic separation of penicillamine was achieved on a dual column packed with ion-exchange resin with automated system (74-76). Friedman (77) described the use of Durrum DC-4A column (48 cm x 1.75 mm) eluted with citrate buffer at gradient pH of 3.25, 4.25, and 7.9 and detected with ninhydrin at 590 nm. Norleucine was used as internal standard and eluted at 120 minutes. Automated injection system was used.

Purdie (78) has successfully used a Technicon, Model NC-1 amino acid analyzer to separate penicillamine on a column of Chromobeads (cation-exchange resin) (150 cm x 0.60 cm) at 60° and it was determined with ninhydrin. A buffer eluent flow rate of 35 ml per hour was used. A rapid and sensitive automated analysis of penicillamine on micro sample in the 1-10 nmole range in physiological fluids was reported (79). Bonnot also achieved a similar separation on a different size column (140 cm x 0.3 cm) with citrate buffer (80).

8.7. Polarographic Analysis

Polarography has been used for the determination of

penicillamine present in biological fluids or as conversion product from penicillins (81, 82).

8.8. Coulometric Method

The use of coulometrically generated mercury (II) for the titration of penicillamine was reported. When pure penicillamine was titrated two equivalence points were obtained (83). The potential at the first equivalence point lies around +80 mV. Titration took place in a 0.4 M acetate buffer solution at a generating current of 5 mA (3 mA before the second equivalence point).

8.9. Titration Method

Billabert (84, 85) used mercury (II) acetate for a titration at pH 6, two equivalence points were found, the first corresponding to formation of a sulfide and the second to formation of a chelate. A 1:1 stoichiometry exists between Hg (II) and penicillamine during titration. Other titrimetric methods, such as titrating with silver (I) ions (86), lead (II) ions (87, 88), alkaline solution after proton displacement reaction (89) and other metallic salts (90) are also reported.

8.10. Optical Purity Analysis

The enantiomers of this drug differ in their efficacy and activity. D-Penicillamine is the enantiomer required for pharmaceutical preparations. The L-enantiomer is toxic and it is also absorbed by the human body more than the D-form (91). Much of the allergic toxicity can be attributed to the use of L-form. It is known that L-penicillamine exerts an anti-vitamin B₆ effect in rats. Since this drug is administered often in doses of up to one gram per day (92), an impurity even of a fraction of a percent of L-isomer may be hazardous. A method for the analysis of the optical purity is necessary.

Cockerill (93) developed a NMR method for assaying the enantiomeric composition of penicillamine. The NMR spectrum of the thiazolidene derivative of penicillamine was taken in the presence of the optically active europium shift reagent, tris-(3-heptafluorobutyryl-d-camphorato)europium. The derivative was prepared from the mixture by successive treatment with acetone-HCl and ethereal diazomethane. The L-enantiomer can be detected at levels of 0.4-0.5% in DL mixture. The shift reagent causes significant separation of

equivalent peaks due to the D- and L- forms of penicillamine e.g. $\Delta\delta = 1.84$ for the methine C-H protons and $\Delta\delta = 1.82$ and 0.65 for the methyl groups of penicillamine.

Gas chromatography-mass spectrometry (94) were used to determine the optical purity of penicillamines. The mixture of penicillamines was desulfurized with Raney nickel, derivatized with pentafluoropropionic anhydride and operated by a gas chromatograph fitted with glass capillaries (2.0 m x 0.3 mm) coated with Chirasil-Val (a novel chiral polysiloxane type stationary phase). The enantiomers were separated isothermally at 110° with a resolution factor $\alpha_{L/D}$ of 1.134. The reliability of the assay was further monitored by mass spectrometry.

The mass spectrometer is set to a resolution of approximately 1000 with ionizing potential of 70 eV ionizing current of 0.8 mA, interface temperature of 220° and ion source temperature of 220°.

9. Metal Complex Formation

The ability of penicillamine to act as chelating agent in therapeutic treatment of Wilson's disease and mercury poison has prompted the extensive investigations by a number of laboratories on the structural chemistry of metal complexes of penicillamine, particularly that of the copper complex (95-101).

A complex was assigned to be Cu (I)/Cu (II) mixed-valence complex based on X-ray diffraction data. Six out of the fourteen copper ions are present in square, planar environment commonly found for Cu (II). The high absorption in the blue region allowed the proposal of some similarity to copper chromophores.

Investigation of complex formation of penicillamine with non-transition and transition metal ions was undertaken by means of analytical potentiometric and spectroscopic techniques such as nuclear magnetic resonance, electron magnetic resonance and circular dichroism. Among the metallic ions examined include nickel (II) (17), zinc (II) (102), lead (II) (102), antimony (III) (103), cobalt (III) (104, 105), indium (III) (106), mercury (II) (90), (107), gold (I) (108-110), and cadmium (II) (111).

Stability constants (112) for metal complexes of D-penicillamine with bivalent metallic ions such as magnesium

(II), calcium (II), manganese (II), iron (II), cobalt (II), nickel (II), copper (II), zinc (II), lead (II), and mercury (II) were reported. These constants were calculated from pH values using known mathematical relations and computer programming.

10. Metallic Salt Formation

The preparation of calcium chloride salt of D-penicillamine (m.p. 300°C) was described. This salt is used as a chelating agent in tablets or capsules (113).

11. Pharmacokinetics and Drug Metabolism

D-Penicillamine is the accepted therapeutic agent for the treatment of Wilson's disease (114, 115), cystinuria (116), lead poisoning (117-119), rheumatoid arthritis (120), and a possible protective agent against radiation (121). Overall, the biochemical actions of D-penicillamine are attributed to its aminothiols properties, i.e., chelating of metals, reaction with carbonyl groups and interference with sulfhydryl disulfide exchange reactions.

The therapeutic value in Wilson's disease as well as the treatment of heavy metal poisoning results from its strong in vivo metal chelating properties. Theories have been advanced for the mechanism of action of penicillamine in rheumatic disease (122, 123). A striking similarity between side effects in rheumatoid arthritis patient caused by gold treatment and penicillamine therapy has been observed. The mode of action of penicillamine in cystinuria is well understood, the mixed disulfide formed with cysteine is more soluble than cystine (124).

Stability constants were determined for penicillamine at simulated biological condition with calcium (II), iron (II), and gold (II). Only iron (III) formed both 1:1 and 1:2 chelates with D-penicillamine (125).

Based on electron spin resonance and chemical studies of penicillamine and copper (II), a mechanism for the action of penicillamine in the treatment of Wilson's disease was proposed by Peisach (125). ESR data indicated that penicillamine initially produces a complex with copper (II) through a microscopically reversible addition of a single ligand atom, i.e., nitrogen to the metal ion. The addition of the second ligand atom - sulfur is quickly accompanied by an electron transfer reaction whereby the metal ion is

reduced to copper (I) and the ligand sulfur is oxidized to a sulfur free radical.

In spite of its wide use in medicine, the pharmacology of D-penicillamine in man is little understood. Gibbs and Walshe (126) studied the fate of orally administered [^{35}S] DL-penicillamine in six cases of Wilson's disease. Wass and Evered (127) have studied the intestinal absorption of L and D-penicillamine in the rat.

Recently, a study on D-penicillamine metabolism in cystinuria, Wilson's disease and rheumatoid arthritis was undertaken. For subjects undergoing treatment for Wilson's disease, cystinuria and rheumatoid arthritis, the total percentage of the D-penicillamine dose excreted was 34, 40, and 34 percent, respectively. D-Penicillamine caused a 32 percent reduction in the urinary excretion of cysteine residues in cystinuria, but 400 percent increase in their excretion in rheumatoid arthritis.

The metabolites of D-penicillamine known to occur in man are D-penicillamine disulfide, the mixed D-penicillamine-cysteine disulfide and S-methyl-D-penicillamine (128). In vivo, the free thiol has also been found in plasma and urine of patients with rheumatoid arthritis after treatment with the drug. It was also found the stable copper-D-penicillamine complex shows a high superoxide reactivity which may be responsible for the therapeutic effects in rheumatoid arthritis.

When incubated with the contents of the stomach and duodenum of the chick, penicillamine was either destroyed or chemically bound and was no longer detected on the chromatograph (129). D-Penicillamine is slowly oxidized by D-amino acid-oxidase. Both enantiomers of penicillamine is desulphydrated by L-cysteine desulphydrase, only the L-isomer inhibits the action of this enzyme (130). In vitro effects reported in the literature include inhibition of DNA and protein synthesis (131), selective inhibition of polio virus growth (132) and prevention of collagen cross linking (133, 134).

12. Toxicity and Side Effects

When administered to mice, penicillamine was tolerated up to 1750 mg/kg intravenously, >5 g/kg orally and exhibited a LD_{50} value of 2300 mg/kg. When administered orally at 50 mg/kg daily for a month, it did not affect the composition

of blood or the functioning of kidneys and liver in rabbits or gastric peristalsis in dogs (135).

Acute sensitivity reactions manifested by fever, rashes (pruritic, morbilli form and urticarial), leukopenia, eosinophilia and thrombocytopenia have been encountered early in the course of therapy. Infrequently, anorexia, nausea and vomiting occurs. Loss of taste for salts and sweets have been observed. Rarely nephrotoxicity has been reported. Extravasation of blood into the skin over pressure joints occurs in some patients after prolonged administration of high doses of penicillamine (136).

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PROBENECID

Abdullah A. Al-Badr and H. A. El-Obeid

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1. Description

1.1 Nomenclature

1.1 1 Chemical Names

4-[(Dipropylamino)-sulfonyl]-benzoic acid.
 p-(Dipropylaminosulfonyl)-benzoic acid.
 p-(Dipropylsulfamyl)-benzoic acid.
 4-(Dipropylsulfamoyl)-benzoic acid.
 4-(Dipropylsulfamyl)-benzoic acid.

1.1 2 Generic Name

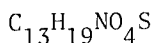
Probenecid

1.1 3 Trade Names

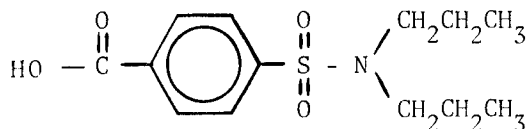
Benacen, Benemid, Benemide, Benn, Probalan, Probecid, Proben, Probenid, Robenecid, Uricocid. It is also an ingredient of Amcil-GC, Benn-C, Colbenemid, Polycillin-PRO, Probampacin, Prolongine, Principen with Probenecil, Robenecol and Robenecid with Colchicine.

1.2 Formulae

1.2 1 Empirical



1.2 2 Structural



1.2 3 Wiswesser Line Notation

QVR DSWN3&3 (1)

1.3 Molecular Weight

285.36

1.4 Elemental Composition

C 54.72%, H 6.71%, N 4.91%, O 22.43%,
 S 11.23%.

1.5 Appearance

White or almost white crystalline powder.

2. Physical Properties

2.1 Melting Point

Melts between 198^o and 200^o (2-4).

2.2 Solubility

Practically insoluble in water, soluble in 25 parts of alcohol (95%), and 12 parts of acetone; also soluble in chloroform and in dilute solutions of alkali hydroxides and of sodium hydrogen carbonate; insoluble in dilute mineral acids (2,4).

2.3 Identification

2.3 1 Infrared Spectroscopic Test

B.P. 1973 (2) and U.S.P. XIX (5) cite the use of infrared absorption spectrum of probenecid as a mean of identification comparing some characteristic absorption bands of the drug. This will be discussed in the infrared spectral properties of the drug.

2.3 2 Ultraviolet Spectroscopic Test

B.P. 1973 (2) and U.S.P. XIX (5) cite the use of ultraviolet absorption spectrum of probenecid in alcohol (95%) as a mean of identification comparing some characteristic absorption maxima of the drug. This will be discussed in the ultraviolet spectral properties of the drug.

2.3 3 Thin Layer Chromatographic Test

B.P. 1973 (2) describes a thin layer chromatographic method for the identification of probenecid comparing the principal spot in the chromatogram of the substance being examined with that of probenecid obtained under identical conditions.

2.3 4 Melting Point Test

B.P. 1973 (2) also uses the melting point of the drug as a mean of identification.

2.4 Spectral Properties

2.4 1 Ultraviolet Spectrum

The ultraviolet spectrum of probenecid in neutral methanol solution in the region of 200 to 350 nm exhibits two maxima at 224 nm and 246 nm and two minima at 214 and 233 nm. The spectrum is shown in Figure 1. According to B.P. 1973 the spectrum of

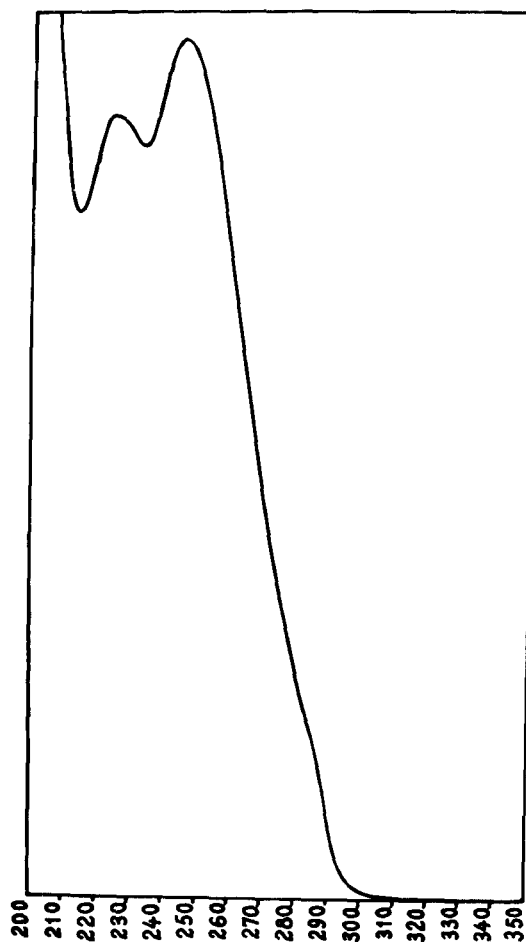


FIGURE 1. Ultraviolet spectrum of probenecid in neutral methanol.

probenecid in ethanol shows maxima at 225 nm and 248 nm (E 1%, 1cm 330).

The ultraviolet absorption spectrum of the drug is used as a mean of identification of probenecid in B.P. 1973 and U.S.P. XIX.

According to the B.P. a 2cm layer of 0.0015% w/v solution in alcohol (95%) exhibits two maxima, at 225 nm and 248 nm; extinction at 248 nm, about 0.99.

2.4 2 Infrared Spectrum

The infrared spectrum of probenecid is shown in Figure 2. The spectrum was obtained from nujol mull. The structural assignments have been correlated with the following band frequencies:

Frequency (cm^{-1})	Assignments
1705	C = O Vibration
1610, 1583	C = C Stretch of aromatic ring.
1295, 1315	Asymmetric-SO ₂ -Stretch
1160	Symmetric-SO ₂ -Stretch
770, 780	CH deformation

The infrared absorption spectrum of the drug is used as a mean of identification of probenecid in both B.P. 1973 and U.S.P. XIX.

2.4 3 Proton Nuclear Magnetic Resonance Spectrum(PMR)

A typical PMR spectrum of probenecid is shown in Figure 3. The sample was dissolved in acetone-d₆. The spectrum was determined on a Varian-T60A NMR spectrometer with TMS as the internal standard. The following structural assignments have been made for Figure 3:

Chemical shift(δ)	Assignments
8.07	Aromatic
3.17	$-\text{CH}_2\text{CH}_2\text{CH}_3$
1.62	$-\text{CH}_2\text{CH}_2\text{CH}_3$
0.87	$-\text{CH}_2\text{CH}_2\text{CH}_3$

2.4 4 ¹³C Nuclear Magnetic Resonance Spectrum (¹³C NMR)

The ¹³C NMR spectrum of probenecid in acetone-d₆ using tetramethylsilane as an internal reference is obtained on a

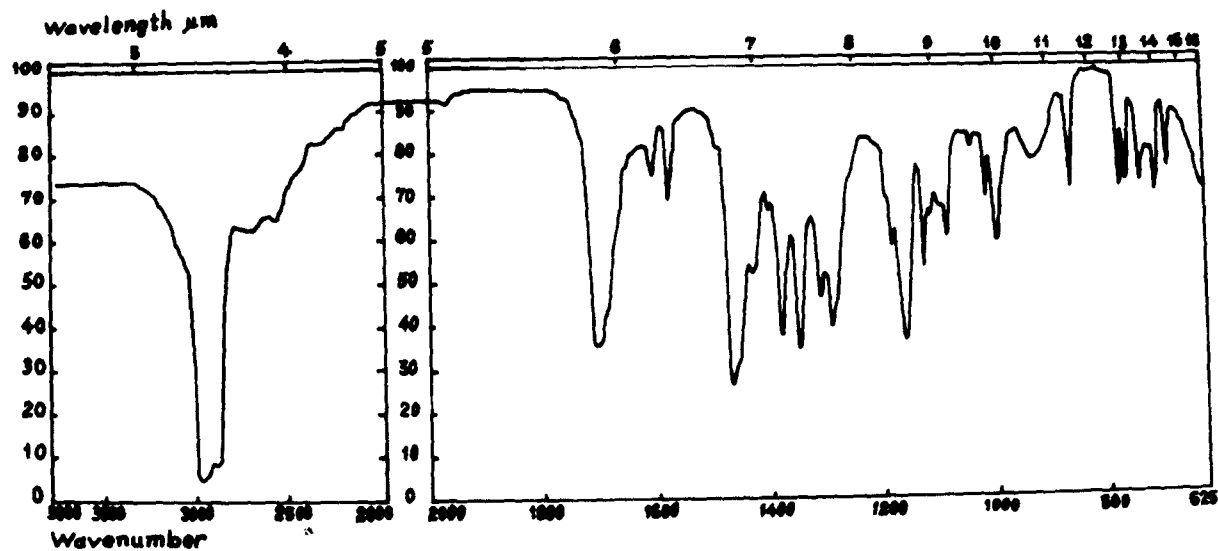


FIGURE 2. Infrared spectrum of probenecid; Nujol Mull.

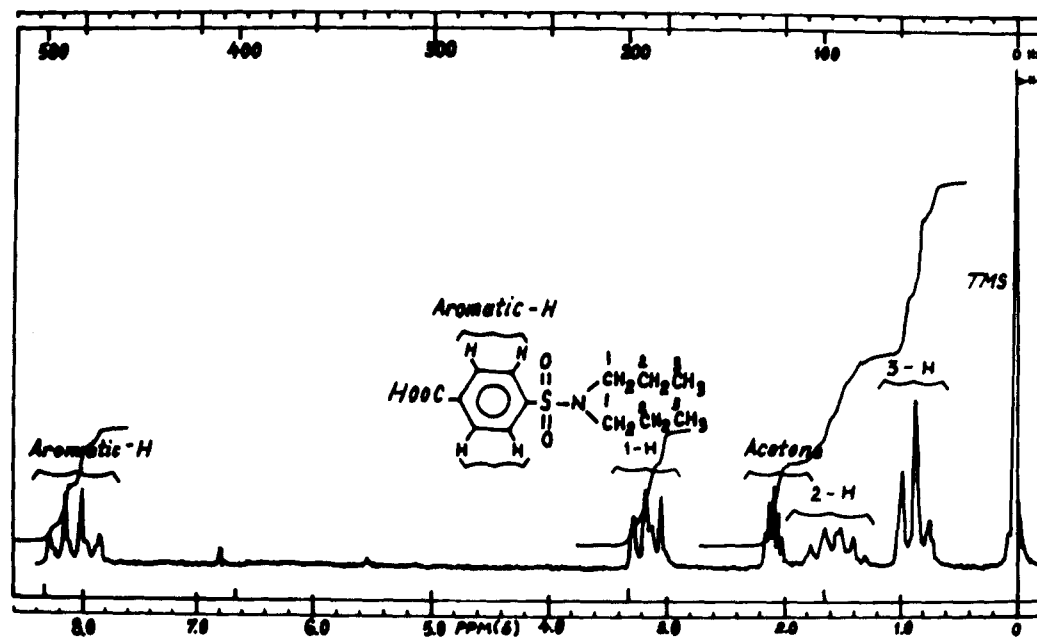


FIGURE 3. PMR Spectrum of probenecid in acetone- d_6 with TMS as internal standard.

Jeol FX100, 100 MHz instrument at an ambient temperature and using 10 mm sample tube. The spectrum is shown in Figure 4 and the carbon chemical shift values, shown in Table 1, are derived from the off-resonance spectrum.

Table 1

^{13}C NMR Characteristics of Probenecid

Carbon No.	Chemical Shift (ppm)	Carbon No.	Chemical Shift (ppm)
1	50.87	8	131.03
2	22.75	9	127.91
3	11.30	10	134.73
4	50.87	11	127.91
5	22.75	12	131.03
6	11.30	13	166.31
7	145.46		

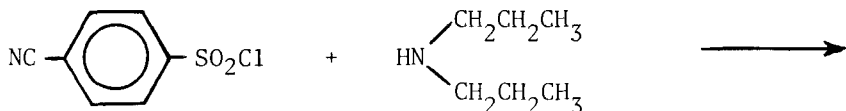
2.4 5 Mass Spectrum and Fragmentometry

The mass spectrum of probenecid (Figure 5), obtained by electron impact ionization, using Nermag GC-Mass spectrometer model R 1010 shows, a molecular ion M^+ at m/e 285 (relative intensity 4.1%) and a base peak at m/e 256. Table 2 shows the proposed fragmentation of probenecid.

3. Synthesis

Probenecid can be synthesized by one of the following methods:

- a) Di-n-propylamine is condensed with p-cyanobenzene-sulfonyl chloride followed by hydrolysis of the nitrile to the carboxylic acid (6).



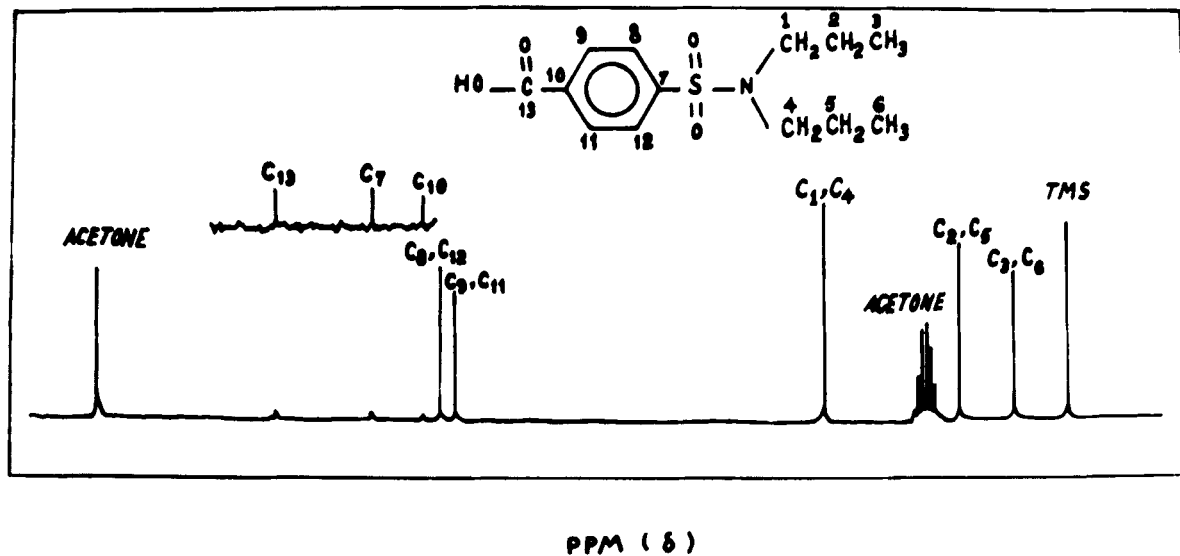


FIGURE 4. ^{13}C NMR Spectrum of probenecid in acetone- d_6 with TMS internal reference.

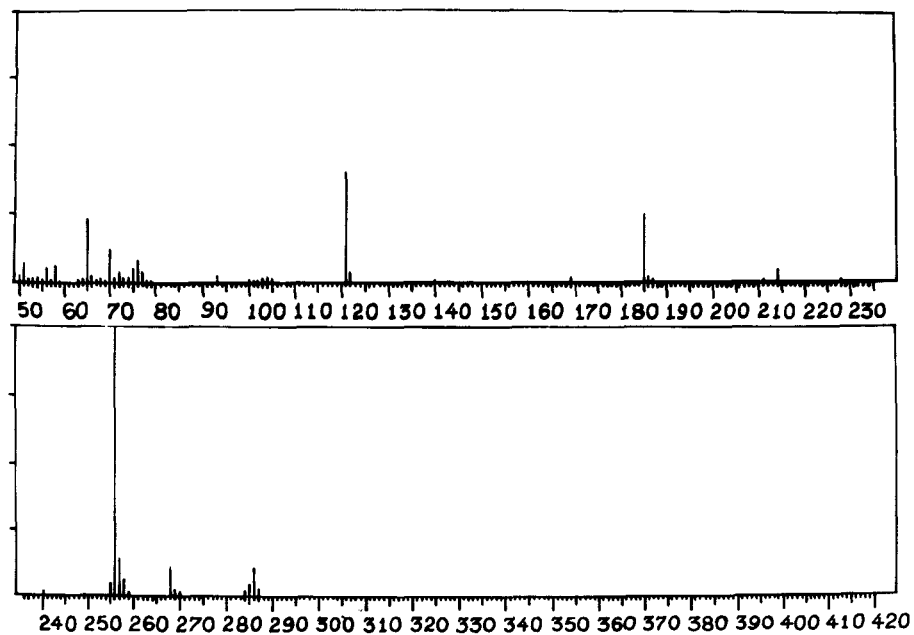
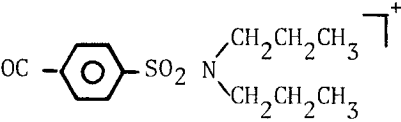
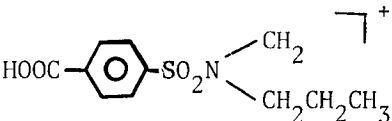
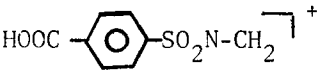
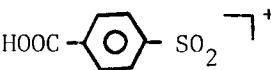

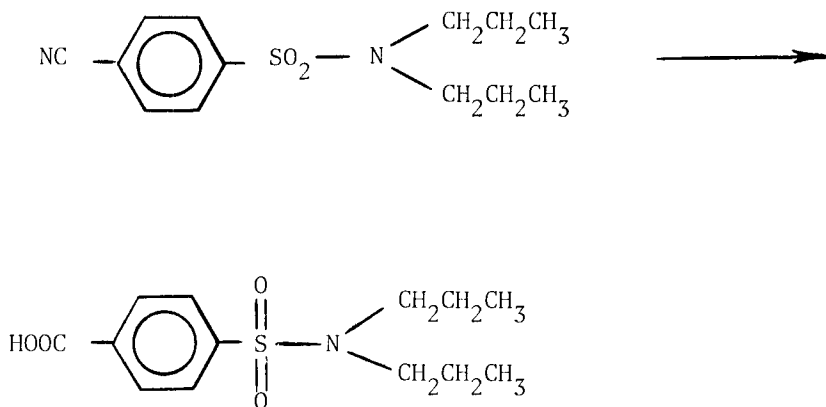


Figure 5. Mass Spectrum of probenecid (EI)

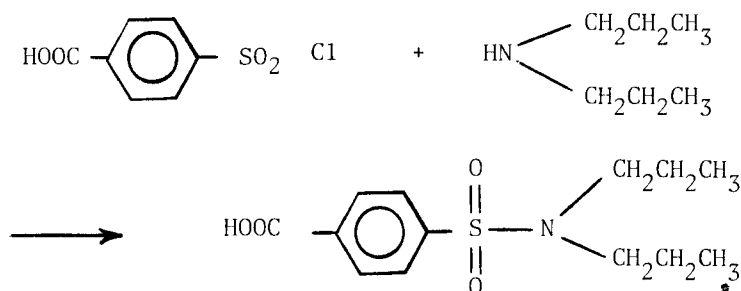
Table 2

Proposed fragmentation of Probenecid (E1)

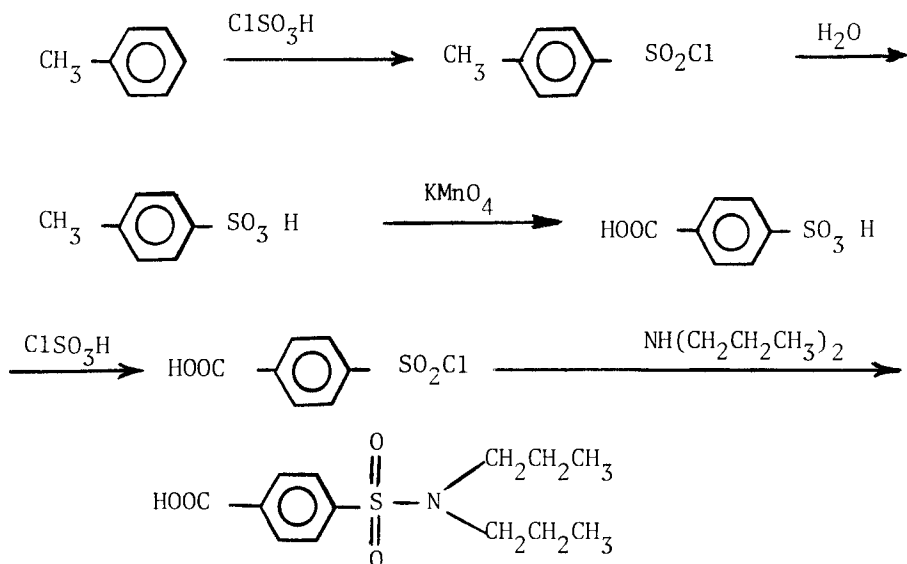
m/e	Relative Intensity%	ion
287	2.0	M+2
286	10.2	M+1
285	4.1	M+
268	10.9	
256	100	
214	4.7	
185	25.1	
121	39.2	



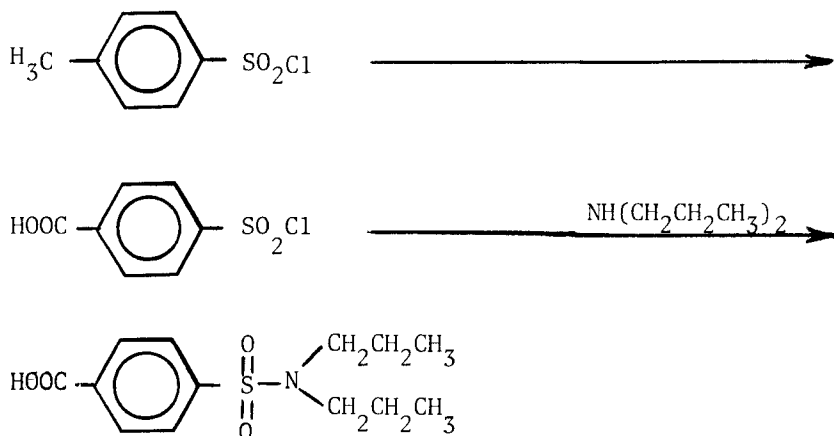
- b) Di-n-propylamine is condensed with p-carboxybenzenesulfonyl chloride (6)



- c) Toluene is treated with chlorosulfonic acid, and the resulting p-toluenesulfonyl chloride is hydrolysed to p-toluenesulfonic acid. Oxidation of the methyl group with potassium permanganate produces a p-carboxybenzenesulfonic acid. This acid is then converted into the corresponding sulfonyl chloride by treatment with chlorosulfonic acid. The resulting sulfonyl chloride is then reacted with di-n-propylamine. Crude probenecid is precipitated by pouring the reaction mixture into cold water. The precipitate is collected, washed and purified by recrystallization from a suitable solvent such as alcohol (4).



- d) p-Toluenesulfonyl chloride yields p-(chlorosulfonyl) benzoic acid. The latter added in portions to a cooled stirred solution of di-n-propylamine in dry acetone, stirring continued for 30 minutes, the suspension concentrated on steam bath to one-third volume, the residue poured into cold water, the solution acidified with dilute HCl, the crude product filtered, decolorized in dilute sodium bicarbonate solution, filtered and the filtrate acidified with an excess of dilute HCl to give probenecid (7)



4. Absorption, Metabolism and Excretion

Probenecid is readily absorbed from the gastrointestinal tract and is extensively bound to plasma proteins. The drug is slowly metabolized and excreted in urine (8). The metabolism of the drug has been extensively studied in several species including humans.

Dayton et.al (9) studied the physiological disposition of the drug including renal clearance in man. The drug was found to be rapidly and almost completely absorbed from the human digestive tract. The half-life, as estimated by rate of plasma level decline of the drug, was the same, whether it was given orally or intravenously; among 9 subjects given 2 gm., the half-life ranged from 4 to 17 hours, less than 5% of the dose was excreted in the urine as unchanged form of the drug in 24 hours. The remainder must have been transferred to unknown metabolites or conjugates.

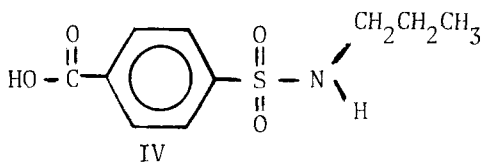
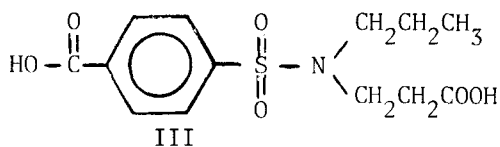
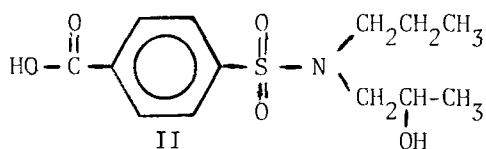
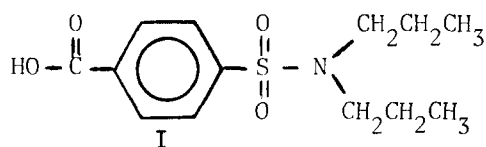
Other studies are published by Perel et.al (10) for the identification and renal excretion of probenecid [I] metabolism in man. The drug given orally to man was excreted in the urine mostly as the probenecid monacylglucuronide [V] (40%) and other metabolites (Figure 6).

The metabolites were excreted mostly in the free form [I], a small amount (3.4%) of the secondary hydroxy [II] and N-depropylated [IV] metabolite being present probably as β -acylglucuronides.

Melethil and Conway (11) have used a gas liquid chromatographic assay to study the excretion of probenecid and its metabolites in the urine of human subjects. The major metabolite, probenecid acylglucuronide [V] accounted for 34-47% of the dose given. Approximately equal amounts (10-15%) of the mono-N-propyl [IV], secondary alcohol [II] and carboxylic acid [III] metabolites were excreted in the unconjugated forms with only traces in the conjugated forms. The urinary excretion of the unchanged drug is dependant on both the pH and flow rate of urine.

Perel et.al (12) also published a study on renal excretion of probenecid acylglucuronide [V] in man. Following a single oral dose to both normal and gouty subjects, about 25% of the probenecid was converted to its acylglucuronide and only a small amount of the drug was excreted unchanged.

Dayton and Perel (13) have studied the metabolism of probenecid in man. Quantitative TLC showed that the drug's metabolism in man yielded monohydroxylated derivative at the secondary [II] (7.2 - 12.5%) and terminal [VI] (1.6-3.7%) positions of propyl side chain and carboxy [III] (6.3 - 9.2%) and N-depropylated [IV] (4.6 - 8.0%) compounds.



Probenecid monoacyl-
glucuronide

V

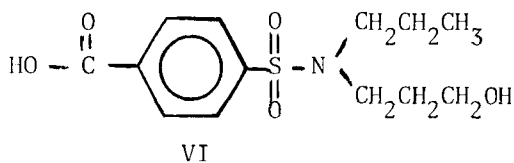


Figure 6. Metabolites of Probenecid.

These metabolites were excreted mainly in the free forms with 3.4% of secondary hydroxy and N-depropylated derivatives probably present as β -acylglucuronides. No phenolic metabolites were formed probably due to electron deactivation of the ring.

Guarino and Schanker (14) studied the biliary excretion of probenecid and its glucuronides. In rat, the drug administered with ligated renal pedicles, readily appeared in the bile as the unchanged drug and as the glucuronide of a metabolite of the drug.

Gigon and Guarino (15) investigated the uptake of probenecid by rat liver slices. The uptake of probenecid- ^{14}C by rat liver was studied by standard tissue slice method. No metabolites of this transport inhibitor accumulated in the slices, but the parent drug and a single metabolite appeared in the incubation media. The metabolite was identified by GLC/MS procedure as the glucuronide of side chain-hydroxylated probenecid.

Conway and Melethil (16) studied the excretion of probenecid and its metabolites in bile and urine of rat. Within 8 hours after i.v. administration of the drug to anesthetized rats, 63.8% of the dose was accounted for in the bile, consisting of (as % of dose) the drug (10%), probenecid glucuronide (15.7%), glucuronide of [II] (20.3%), glucuronide of [VI] (14.2%) and p-(N-propyl-N-2-carboxyethyl sulphamoyl) benzoic acid [III] (3.6%). Ligation of the renal pedicles increased excretion of all metabolites to a total recovery of 86.6%. p-(N-Propylsulphamoyl) benzoic acid [IV], unconjugated N-2-hydroxy propyl [II], N-3-hydroxypropyl [VI], and N-2-carboethoxy derivative [III] of the drug. Neither the drug nor its glucuronide were found in the urine of probenecid-treated rats.

Cunningham et.al (17) have studied the in vitro metabolism of probenecid in rat, mouse and human liver preparations and the factors affecting the site of oxidation. The metabolism was limited to the side chain, involved mono-N-depropylation and hydroxylation in the 2 and 3 positions (3-hydroxyprobenecid was in part converted to the carboxy metabolite). Acylglucuronide conjugation was minimal. Balogh et.al (18) have reported the pharmacokinetics of probenecid during the neonatal period. The drug was reported to have eliminated from the serum more slowly in 5- and 15-days old rats than in older rats. The drug was eliminated entirely in the form of metabolites in which four were detected in urine. The drug was excreted in both urine and bile. Alkalinization of the urine accelerated the drug excretion in adult rats.

5. Methods of Analysis

5.1 Titrimetric Method

B.P. 1973 (2) and U.S.P. XIX (5) describe for the determination of probenecid powder a titrimetric assay method based on neutralizing the carboxylic acid group of the drug with standard alkali.

5.2 Spectrophotometric Methods

5.2 1 Ultraviolet Spectrophotometric Method

- a) B.P. 1973 (2) uses for the assay of probenecid tablets a spectrophotometric method which is based on the extraction of drug with acidified alcohol and measuring the extinction of a 1-cm layer of the resulting solution at a maximum at about 248 nm. The amount of the drug is calculated taking 332 as the value of E (1%, 1cm) at the maximum at about 248 nm.
- b) The spectrophotometric method of U.S.P XIX (5) for the determination of probenecid in tablets utilizes chloroform as the solvent for extraction and the absorbance is determined in 1-cm cells at the wavelength of maximum absorbance at about 257 nm.
- c) Tillson et.al (19, 20) described a method for the determination of probenecid in plasma and urine. According to this procedure the drug is extracted into chloroform from the acidified fluid, then re-extracted into dilute aqueous sodium hydroxide solution and determined electrophotometrically in the ultraviolet region at 242.5 nm. The extinction coefficient in g/l is 36.43. The recovery is 95% or better. A reagent blank is run alongside and should not have an optical density greater than 0.50. As the method is not specific, blanks should be obtained from each patient before the drug is administered.

5.2 2 Colorimetric Methods

- a) Tillson et.al (19) reported a colorimetric method for the estimation of the drug in body fluids suitable for clinical purposes as it is unaffected by most medicaments and endogenous metabolic products. In

this method the drug is extracted into chloroform from the acidified fluid, and the separated chloroform layer is shaken with an aqueous solution of methylene blue. The colored salt formed is soluble in chloroform and the amount formed can be determined colorimetrically at 635 nm.

- b) Beltagy et.al (21) also reported a similar colorimetric method for the determination of probenecid. It involves the treatment of the drug with a $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer (pH 6.8), methylene blue and chloroform, then measuring the absorbance of the chloroform extract of the complex. The maximum absorption (λ_{max}) was 640 nm. This method is useful for small amounts of the drug (≥ 0.1 mg).
- c) Wahbi et.al (22) published a colorimetric method for the estimation of probenecid in tablets which is based on the use of basic fuchsin as an ion-pairing reagent for the acidic drug. According to this method the active drug was extracted from tablets, and the drug aqueous solution at pH 7 treated with aqueous basic-fuchsin. The salt was extracted into chloroform and the absorbance measured at 550 nm, and the concentration of the drug calculated from the corresponding calibration graph. Beer's Law is obeyed for 20 to 80 ug of probenecid in 50 ml of chloroform solution. The method is applicable to the bulk drug or to tablets.
- d) A technique for identifying probenecid in tablet dosage form by spot test on paper had been utilized by Cooper (23). The technique was based on the reaction of the drug with ferric iron and dimethylaminobenzaldehyde to produce colored products. The two reagents used for color production are, a slightly acidified aqueous solution of ferric chloride and an acidified ethanolic solution of p-dimethylaminobenzaldehyde (Ehrlich's reagent). The interfering factors and the color obtained by reaction of these reagents with probenecid are shown in Table 3.

Table 3

Conditions for Spot Identification of Probenecid

Conditions	Ferric Chloride Reagent	Ehrlich Reagent
Blank-Reagents dried on paper	pale yellow	pale yellow
5% KOH	ochre	no color
10% HCl	no color	no color
Excipients	no color	no color
Probenecid + acidified reagent	ochre	primose

5.2 3 Spectrophotofluorometric Method

A spectrophotofluorometric assay for probenecid was presented by Cunningham et.al (24). The method was based on conversion of the drug to a fluorescent anthranilic acid derivative. Blood plasma or cerebrospinal fluid was acidified with 3 N-hydrochloric acid and extracted with 1,2-dichloroethane. An aliquot of the organic layer was evaporated to dryness with air stream at room temperature and the residue was converted to the anthranilic acid derivative. The fluorescence is measured at the emission wavelength of 415 nm with excitation at 340 nm. The fluorescence is directly proportional to the amount of probenecid from 4 to 100 μg . The assay was especially applicable with clean biological fluids (such as cerebrospinal fluid and blood) and offered several fold greater sensitivity than the commonly used ultraviolet methods. Pentobarbital, salicylates and metabolites of probenecid did not interfere.

5.2 4 Molecular Emission Cavity Method

Al-Abachi (25) described a molecular emission cavity analytical (MECA) method for the determination of sulfur containing drugs, amino acids and proteins from their S_2 emission at 384 nm. Probenecid gave t_s of 1.45, calibration range of 5-100 ng^mS , limit of detection of 2.0 ng S and coefficient variation of 3.10%.

5.2 5 Nuclear Magnetic Resonance Spectrometric Method

An NMR procedure is described by which probenecid is determined in pure and tablet formulation. The method is rapid, accurate and precise (26) (s.d. 0.73 and 1.11), and also provide a method of identification of the drug. The spectrum was run in acetone- d_6 with the use of maleic acid as an internal standard using the aromatic protons of probenecid at 8.07 ppm and the singlet at 6.42 ppm of the methylene protons of the maleic acid (the internal standard) as the criteria for analysis.

5.2 6 Mass Spectrometric Method

Probenecid was assayed by selective ion monitoring (m/e 388) using *m*-(diisobutylsulfonyl) benzoic acid as an internal standard (27). The standard curve was linear over the range 4-20 μ g probenecid and the percentage recovery of the drug was 92.7%. The method was applied for determination of probenecid in human cerebrospinal fluid and could be incorporated into the selective ion monitoring procedure used to quantitate other acidic and neutral compounds on such samples.

5.3 Chromatographic Methods

5.3 1 Thin Layer Chromatography (TLC)

- a) A TLC procedure appears in B.P. 1973 (2) for the identification of probenecid and to test for related substances. The method utilizes gel G/UV 254 as the coating substance and a mixture of alcohol and ammonia as the mobile phase. After drying, the chromatogram is examined under ultraviolet lamp having a maximum output at about 254 nm.
- b) Gecgil (28) reported a TLC method for the identification of probenecid and related sulfonamide-type diuretics in tablets and suppositories. The drugs are extracted from tablets and other pharmaceutical preparations with ethanol or acetone and the extracts analysed by TLC on silica gel using ethanol-chloroform-heptane (1:1:1) as solvent. The

spots are visible in UV light and give color reactions when sprayed with pentacyanonitrosylferrate-III (Sodium nitroprusside) in NaOH, or with the Van Urg reagent (dimethylaminobenzaldehyde in HCl and 95% ethanol), or Bratton-Marshall reagent (5% NaNO_2 + 1 % N-(1-naphthyl)ethylenediamine-2HCl) or with $\text{Hg}_2(\text{NO}_2)_2$ (obtained by treating an excess of Hg with HNO_3). The R_f value of probenecid is 0.98.

- c) Another TLC method (29) is used to identify nonmercurial diuretics on silica gel using toluene, xylene, 1,4-dioxane, isopropyl alcohol, 25% NH_4OH (10:10:30:30:10) and propyl alcohol-ethyl acetate-water-25% NH_4OH (50:10:30:10) as solvents and a 5:1 mixture of 10% CaSO_4 and 2% NH_4OH as the spraying agent which gives after heating to 110° for 10 min, blue or brown color reactions with substances containing amido groups. With the two solvents probenecid had R_f values of 0.5 and 0.78 respectively.

5.3 2 Gas-Liquid Chromatography (GLC)

- a) A method (30) is reported for the determination of probenecid in cerebrospinal fluid which employs gas-liquid chromatography and electron capture detection (GLC-ECD). The drug is reacted with trifluoroacetic anhydride and pentafluoropropanol to obtain pentafluoropropionate ester. The high sensitivity of ECD for this derivative allows for the determination of probenecid in as little as 20 μl of cerebrospinal fluid.
- b) A quantitative method for the determination of the drug in biological fluids is also described by Zacchei and Weidner (31). The method employs the N-dibutyl analog of probenecid as the internal standard to be added to plasma or urine samples followed by acidification and extraction into benzene. The acids thus extracted were converted to methyl esters by reaction with ethereal diazomethane and analysed by gas chromatography. As little as 250 μg of probenecid could be detected in 1 ml of plasma.

- c) A gas-liquid chromatographic assay for probenecid and its metabolites and the utilization of this method for the examination of the disposition of the drug in rats and human subjects is developed by Melethil (32).
- d) A sensitive gas chromatographic (GC) method for the measurement of probenecid in biological fluids is described by Sabih (33). The method involves the conversion of probenecid to its methyl ester by treatment with dimethylsulfate under basic conditions. Analysis was performed on Gas Chrom. Q coated with DC-200. The major metabolic product of probenecid, the glucuronide conjugate of the unchanged drug, was identified by combined GC and high resolution mass spectrometry following enzymic hydrolysis.
- e) Conway and Melethil (34) described a gas chromatographic assay method for probenecid and its metabolites in biological fluids. The method is capable of monitoring concentrations of the drug and its metabolites in urine at least 3 days after oral injection of 0.5 g of probenecid. According to the method the free acids, or the acids liberated by acid hydrolysis, were extracted from acidified urine with methylene chloride. After the addition of N, N-dibenzyl-2,5-dimethylbenzenesulfonamide as internal standard, the acids were determined by GLC on a stainless steel column (6 ft x 0.125 in.) packed with 10% of OV-1 on Chromosorb W-HP (80 to 100 mesh) and operated at 250°, with N as carrier gas (23 ml min⁻¹) and a flame ionization detector. Calibration graphs were rectilinear for 25 to 700 µg of probenecid or metabolite. Recoveries of the N-monopropyl, N-carboxyethyl and N-(2-hydroxypropyl) -metabolites were in the range of 10 to 400 µg per 2 ml of urine.

5.3 3 High Pressure Liquid Chromatography (HPLC)

- a) An HPLC method (35) for the determination of probenecid in oral suspensions of ampicillin gave good intra- and interlaboratory reproducibility and accuracy; and interference from excipients and flavour ingredients was

eliminated. Average recoveries ranged from 95.2 to 99.1% with coefficient of variation from 1.63 to 4.9%. Instead of an internal standard, use of a closed-loop injection system (or other means of obtaining a constant injection volume) is recommended. It is also recommended that this method replaces present extraction methods for oral suspensions in the Code of Federal Regulations and other official compendia.

- b) A high performance liquid chromatography method (36) was also used to determine probenecid in serum. Satisfactory results were obtained over the normal therapeutic range of drug (≤ 150 mg/ml). The sensitivity of the procedure was about 1 ug/ml. Neither the metabolites of probenecid nor epicillin, which is frequently co-administered with the drug, interfered with the chromatographic behaviour of probenecid.

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SALBUTAMOL

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and S. E. Ibrahim*

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1. Description

1.1 Nomenclature

1.11 Chemical Names

α^1 -[(tert - Butylamino) methyl]-4-hydroxy-m-xylene-
 α - α' -diol
 N-tert-Butyl-2-(4-hydroxy-3-hydroxymethylphenyl)-
 2-hydroxylamine
 2-(tert-Butylamino)-1-(4-hydroxy-3-hydroxymethyl-
 phenyl)-ethanol
 4-Hydroxy-3-hydroxymethyl- α -[(tert-butylamino)
 methyl]-benzyl alcohol
 α^1 -[(1-tert - Butylamino) methyl]-4-hydroxy-m-
 xylene- α - α' -diol
 1-(4-hydroxy-3-hydroxyphenyl)-2-tert -butylamino-
 ethanol.

1.12 Generic Names

Salbutamol, Albuterol, AH 3365, Sch 13949W.

1.13 Trade Names

Aerolin, Broncovaleas, Sultanol, Venetlin,
 Ventolin.

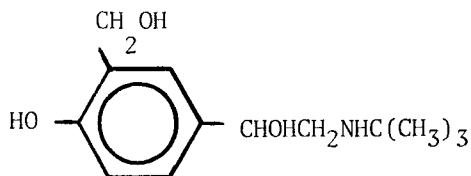
1.14 Registry No. CA 18559-94-9.

1.2 Formulae

1.21 Empirical

$C_{13}H_{21}N O_3$ (base).

1.22 Structural



1.3 Molecular Weight

239.31

1.4 Elemental Composition

C, 65.24 H, 8.85 N, 5.85 O, 20.06

1.5 Appearance

White crystalline powder from ethanol-ethyl acetate or ethyl acetate-cyclohexane (1). The powder is odorless and almost tasteless.

2. Physical Properties

2.1 Crystal Properties

2.11 Crystallinity and X-ray Crystallography

Beale and Stephenson (2) had determined the X-ray crystallographic structure of certain broncho-dilators which included salbutamol, they reported that the drug had conformational characteristics of a β -adrenoreceptor stimulant. Furthermore, Beale and Grainger (3) had published the X-ray analysis of salbutamol and found that it belongs to the space group $Pbca$ with a 21.654 [10] and b 8.798 [4] c 14.565 [7] Å ; $Z = 8$; $d[\text{obsd}] = 1.15$, $d[\text{calcd}] = 1.15$.

The benzene ring is inclined at $74.5[2]^\circ$ to the plane of the $C[7]-C[8]-N-C[9]$ atoms. It was reported that the tert. butyl group is the opposite side of the salbutamol molecule to the amino and the hydroxy groups. The bond distances and angles as well as the atomic position coordinates were discussed.

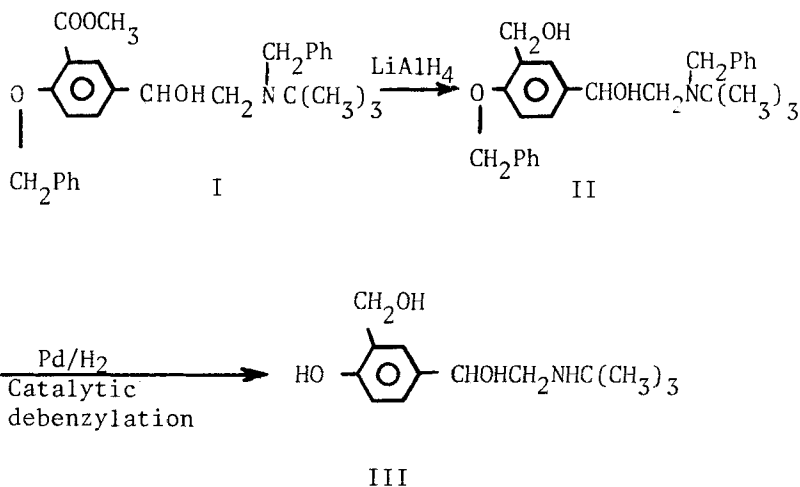
2.12 Melting Point

The British Pharmacopoea (B.P.) 1973 (4) specifies a m.p. of salbutamol at about 156° . Other melting ranges of salbutamol are given below:

Melting range, C°	Ref.
151-152	(1)
157-158	(1)

2.2 Optical Rotation and Circular Dichroism

Hartley and Middlemiss (5) had separated the two optical isomers of salbutamol by forming a benzyl ether II from its synthetic precursor I followed by the precipitation of II with either the (+) or (-) di-p-toluoyltartaric acid. In each case only one isomer formed a crystalline salt and the antipode was recovered from the mother liquor. The purified salts were neutralized and liberated to yield the required optical isomer of salbutamol III.



The circular dichroism (CD) spectral studies indicated that the (-)-isomer had the R-configuration.

The (-)-salbutamol showed a clear negative cotton effect at 276-280 nm. At a lower wavelength, 220-230 nm, the curves tended towards a further negative peak although this was somewhat masked by the high aromatic absorption (5). The physical properties of the optical isomers are shown below:

	m.p.C°	$[\alpha]_D$	C.D.
R-(-) Salbutamol acetate monomethanolate.	144.3	-36.9 (C, 0.27 H ₂ O)	(C, 0.37) $[\theta]_{260}$ 0 $[\theta]_{280}$ -269 (max) $[\theta]_{295}$ 0
S(+) Salbutamol acetate monomethanolate	145.7	+36.9 (C, 0.23)	(C, 0.23) $[\theta]_{260}$ 0 $[\theta]_{278}$ +683 (max) $[\theta]_{295}$ 0

2.3 Solubility

Salbutamol is soluble 1 in 70 of water and 1 in 25 ethanol, slightly soluble in ether and soluble in most organic solvents (4).

2.4 Identification

The following tests are cited from B.P. 1973 (4):-

- The infrared absorption spectrum exhibits maxima which are only at the same wavelength as, and, have similar relative intensities to, those of the spectrum of salbutamol A.S.
- The light absorption, in the range 230-250 nm, of a 2 cm layer of 0.004% w/v solution in 0.1 N HCl exhibits a maximum only at 276 nm; extinction at 276 nm about 0.56.
- Dissolve 10 mg in 50 ml of 2% w/v solution of borax, add 1 ml of 3% w/v solution of 4-aminophenazone, 10 ml 2% w/v solution of potassium ferricyanide and 10 ml of chloroform, shake and allow to separate, an orange-red color develops in the chloroform layer.
- To 2 ml of 1% w/v solution, add 2 drops of FeCl₃ T.S., a reddish-orange color develops which does not change on the addition of sodium hydrogen carbonate solution.

Furthermore, salbutamol gives a pale-yellow color with Marquis-reagent (6).

Other color tests include the following:-

Reagent	Color	Sensitivity
H ₂ SO ₄	Yellow	1.0 µg
H ₂ SO ₄ /HCHO	Pale — yellow	1.0 µg
Ammonium Molybdate	Green → yellow	0.1 µg
Ammonium Vanadate	Blue rim → brown rim	0.1 µg
Vitali's test	Pale yellow-bright orange	0.1 µg

Salbutamol can be identified by forming irregular plates with gold-bromide solution, sensitivity 1 : 100 (6).

2.5 Spectral Properties

2.51 Ultraviolet Spectrum

Salbutamol sulphate in 0.1 N hydrochloric acid shows maxima at 225 nm ($E_{1\%}^{1\text{cm}}$, 1 cm 310) and 276 nm ($E_{1\%}^{1\text{cm}}$, 1 cm 60) (6). In 95% neutral ethanol, salbutamol base absorbs ultraviolet radiation at 276 nm and 278 nm as shown in Figure 1. The ultraviolet spectra of salbutamol at pH 2 (λ_{max} 276 nm) and pH 12 (λ_{max} 296 nm) were reported by Evans *et al* (7) for the comparison with the metabolite isolated in man which does not show bathochromic shift as shown in Figure 2. The bathochromic shift observed by changing the pH from acid to alkaline condition was accompanied by hyperchromic effect.

2.52 Fluorescent Properties

The fluorescent properties of salbutamol were studied by Evans *et al* (7) using Fluorispic 100E spectrophotofluorometer at pH 7. The maximum wave length of excitation and emission reported were 230 nm and 312 nm respectively.

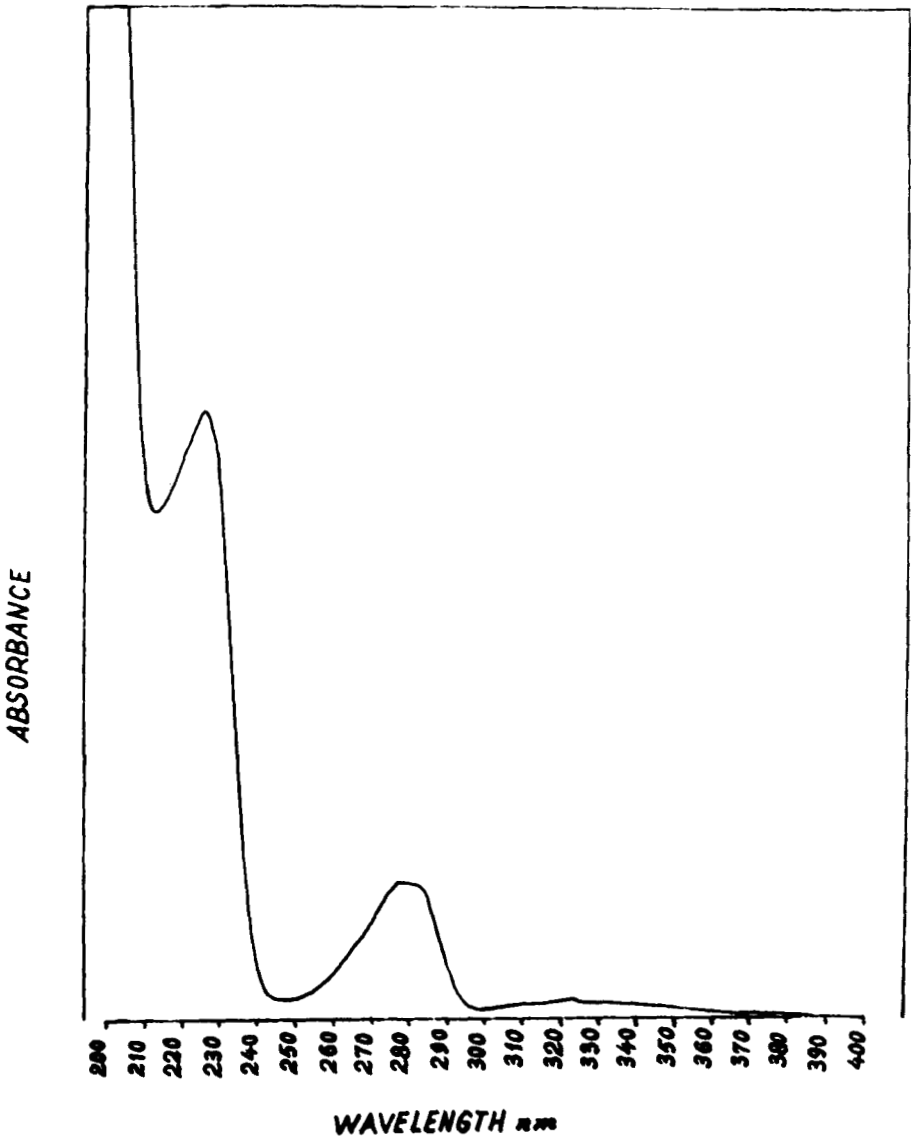


Fig. 1: Ultraviolet spectrum of salbutamol in 95% ethanol.

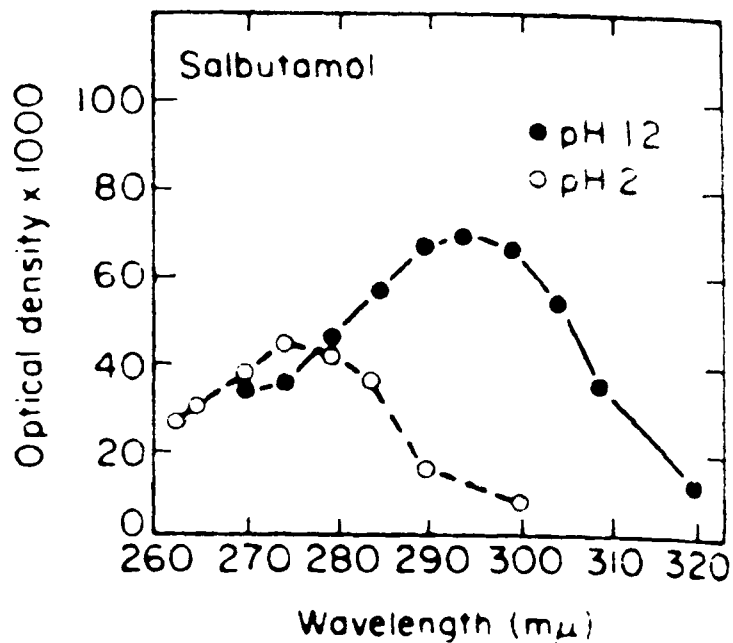
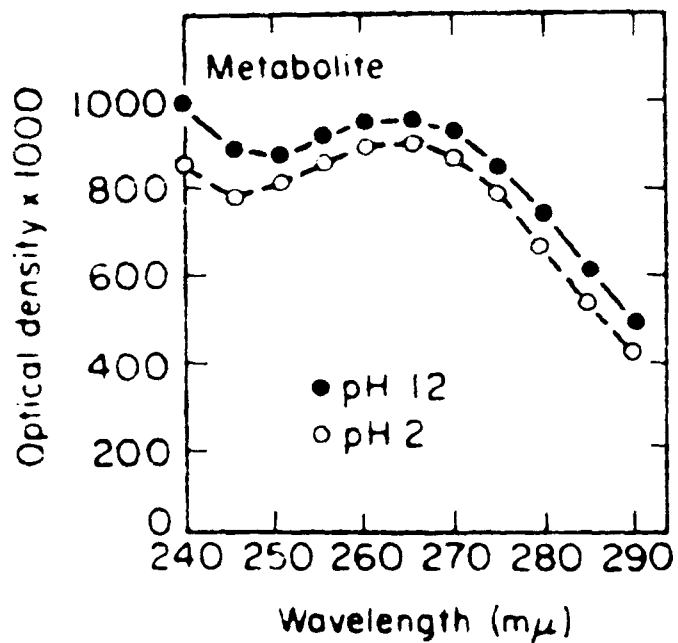


Fig. 2. The ultraviolet spectrum of salbutamol and metabolite at pH₂ and pH₁₂.

2.53 Infrared Spectrum

The infrared spectrum of salbutamol base in nujol mull is given in Figure 3. Major band assignments are as follows :-

Frequency cm^{-1}	Assignment.
3320, 3200, 3160	Phenolic OH, alcoholic OH and NH stretching bands.
1610	Aromatic ring C=C stretching.
1370, 1270, 1190.	Phenolic C - O stretching.
1150 and lower.	Aromatic CH bending.

Other fingerprint bands characteristic to salbutamol (6) (determined in KBr disc), are: 1038, 1075, 1263, 1228 and 1333 cm^{-1} , as shown in Figure 4.

2.54 Nuclear Magnetic Resonance Spectrum

The 60 MHz PMR spectrum of salbutamol base in deuterated dimethylsulphoxide is shown in Figure 5. The spectrum was determined in Varian T60 A NMR spectrometer with TMS as the internal standard. Assignments of the bands are as follows :-

Chemical shift (ppm)	Assignment
Singlet at 1.0	$\text{-C(CH}_3)_3$
Doublet centered at 2.6	$\begin{array}{c} \text{OH} \\ \\ \text{-CH-CH}_2\text{-N-} \end{array}$
Singlet (due to HDO signal) overlapped by a triplet centered at 4.50*.	$\begin{array}{c} \text{OH} \\ \\ \text{-CH-CH}_2\text{-N-} \end{array}$
Singlet at 5.03	$\text{-CH}_2\text{ OH (benzylic protons).}$

*When salbutamol base was determined in pyridine, the overlapping due to HDO signal was resolved to give a triplet at 3.97 ppm for $\text{-CHOH CH}_2\text{-N.}$

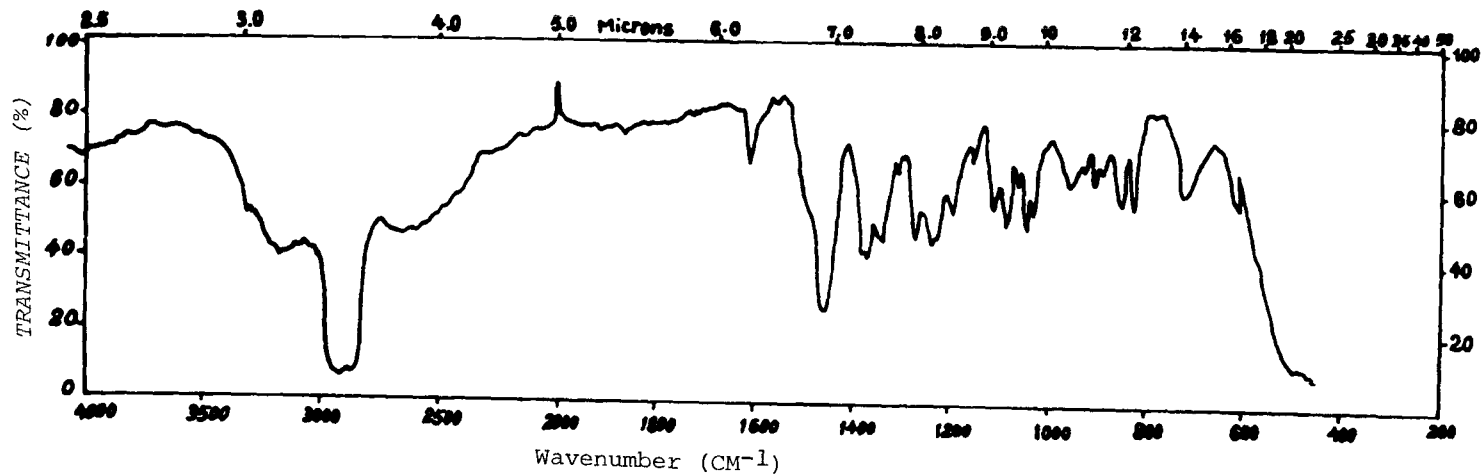
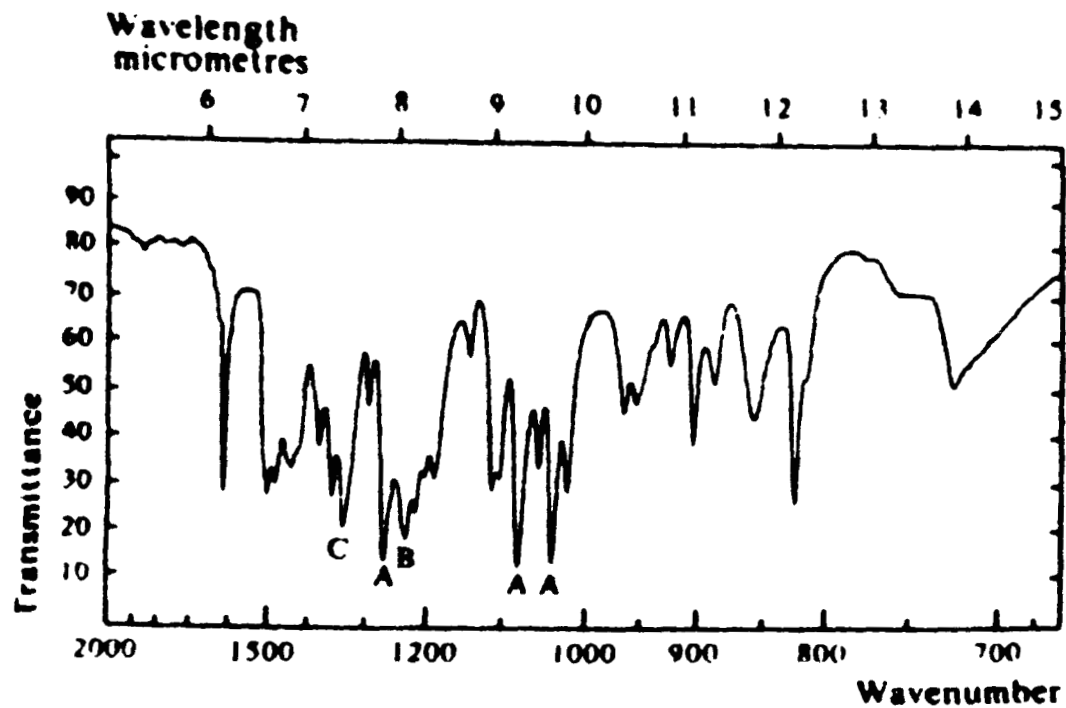


Fig. 3. Infrared spectrum of salbutamol in nujol mull.



A 1038 or 1075 or 1263, B 1228, C 1333

Fig. 4. Fingerprints bands of salbutamol (KBr disc).

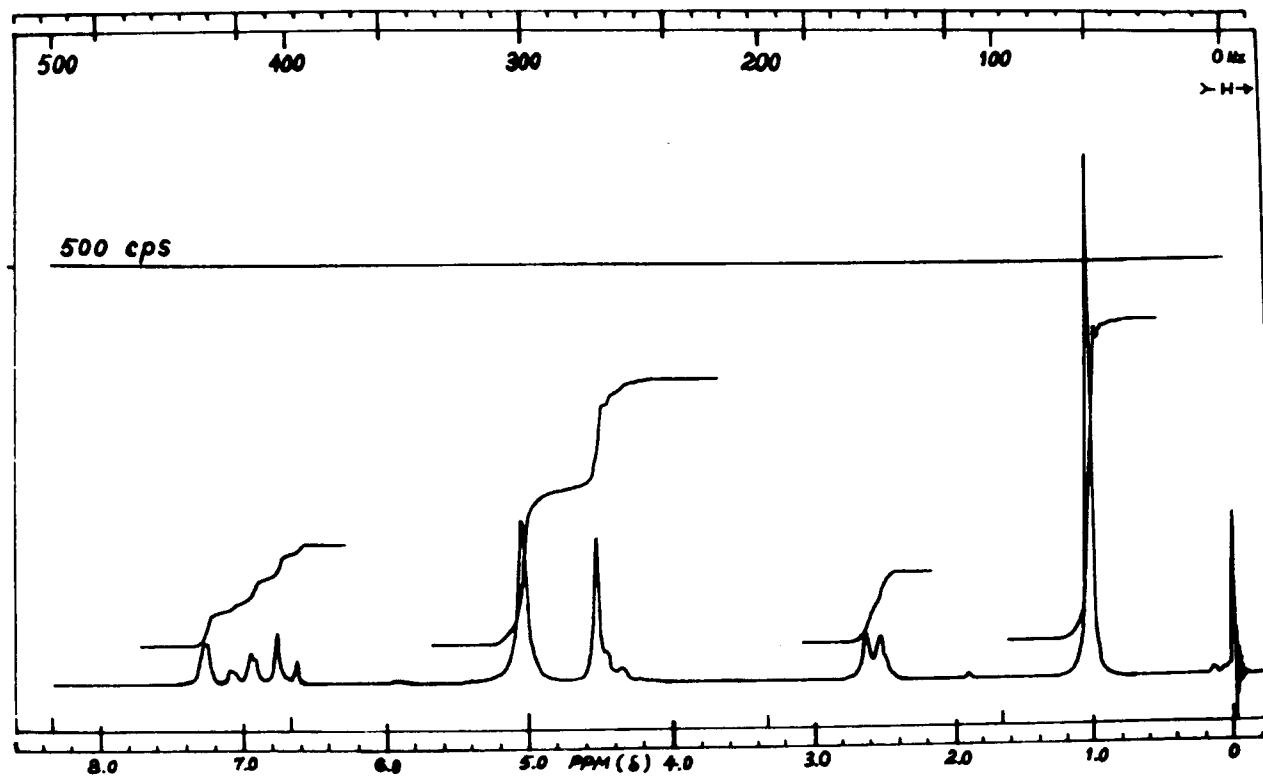


Fig. 5: NMR spectrum of salbutamol containing TMS as internal standard.

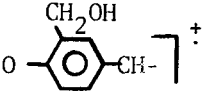
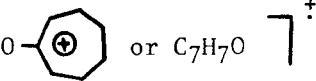
Multiplet between 6.66-7.27 Aromatic H₂, H₅ and H₆ of the aromatic ring.

Parfitt *et al* (8) have determined the optical purities of salbutamol among other substances by NMR using the chiral lanthanide shift reagent (CLSR) by applying the base line technique. The molecular conformation of several adrenergic and β -adrenolytic substances had been studied by NMR (9).

2.55 Mass Spectrum and Fragmentometry

The mass spectrum of salbutamol base obtained by electron-impact ionization, Figure 6, shows a molecular ion M⁺ at m/e 239 (relative intensity 4.3) which becomes pronounced when determined by chemical ionisation (isobutane gas) as shown in Figure 7. The medium resolution EI/MS was determined by direct inlet to Ribermag -10 Mass Spectrometer. The proposed fragmentation ions given in Table 1 are consistent with the salbutamol structure.

Table (1)

Mass (m/e)	Relative Intensity	Ions
241	3.2	M ⁺ 2
240	9.8	M ⁺ 1
239	4.3	M ⁺
206	10.0	M ⁺ 1 - H ₂ O & HO [•]
135	21.1	
107	14.2	 or C ₇ H ₇ O
86	100.0	-CH ₂ -NH C (CH ₃) ₃
57	55.4	-C (CH ₃) ₃

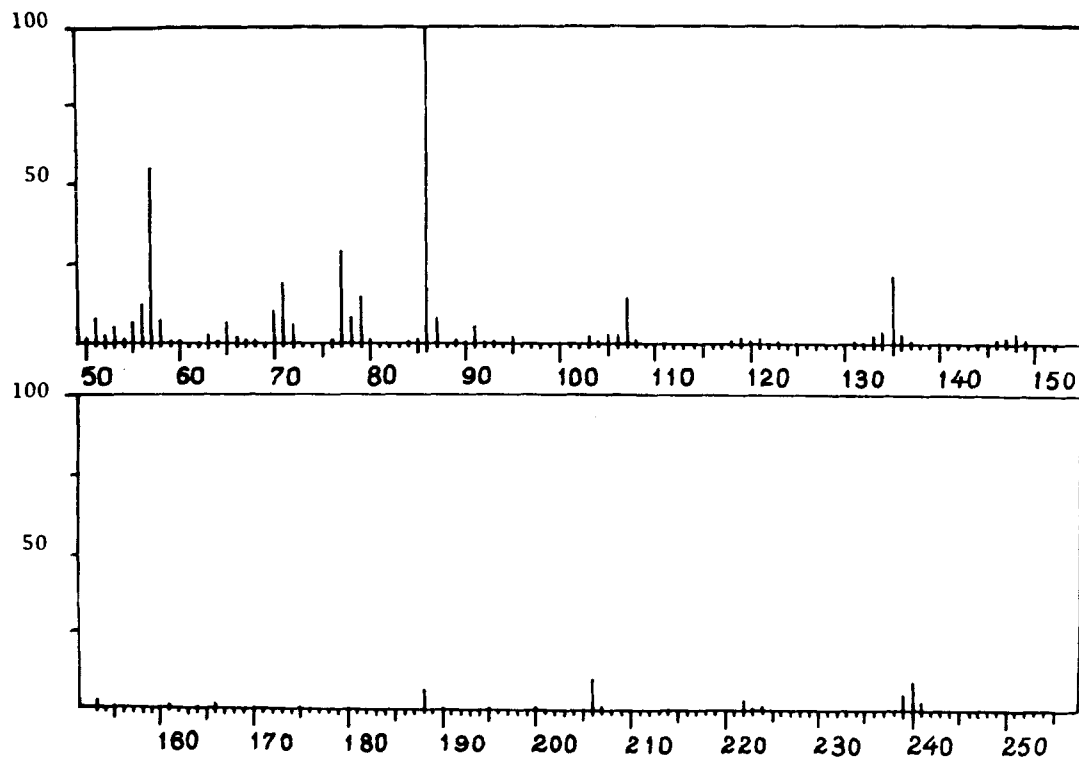


Fig. 6: Mass spectrum of salbutamol (EI).

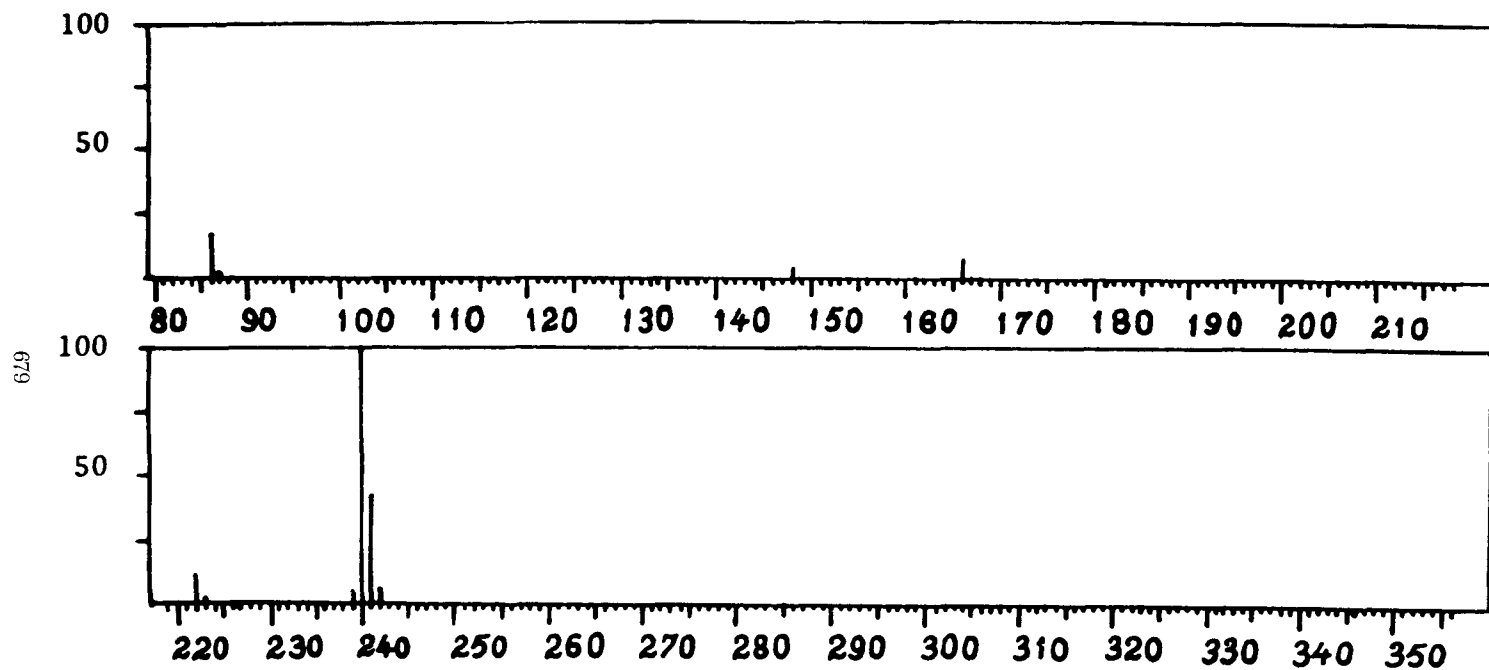
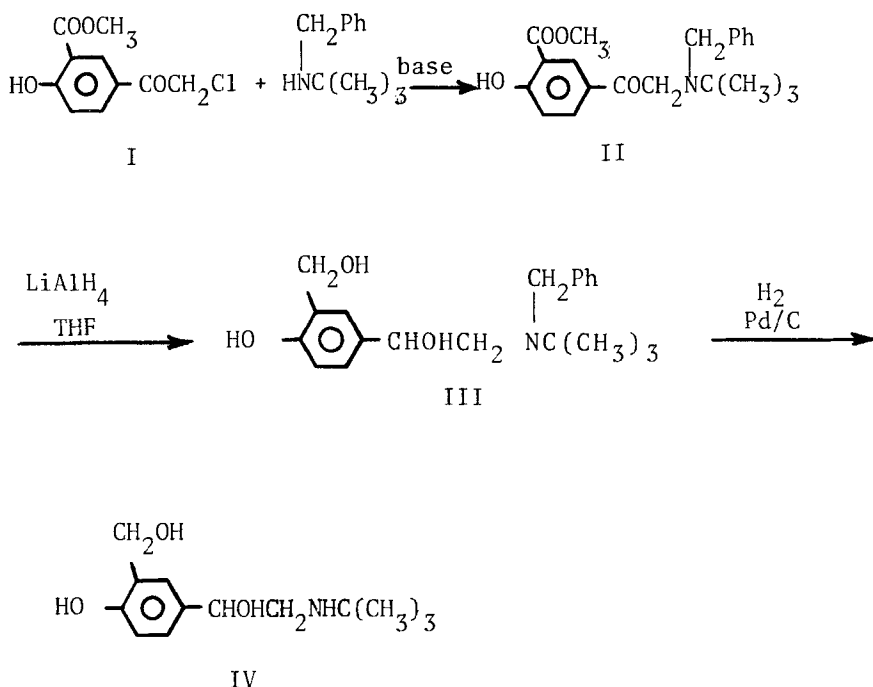


Fig. 7: Mass spectrum of salbutamol (CI-isobutane C_4H_{10}).

3. Synthesis

Several methods have been published and patented for the synthesis of salbutamol. They are summarized as follows:

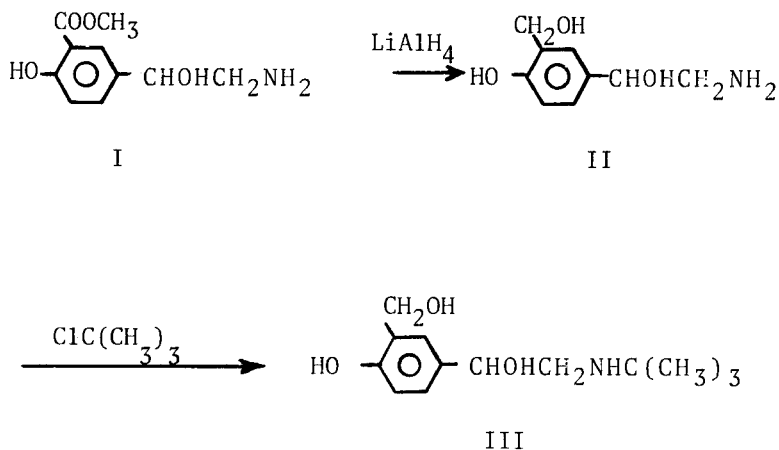
- a) Salbutamol has been prepared by Lunts *et al.* (10) starting from the appropriate acetophenone derivative I by condensation with tertiary butyl benzylamine to give II as shown in Scheme 1. The ketonic ester II is



Scheme 1.

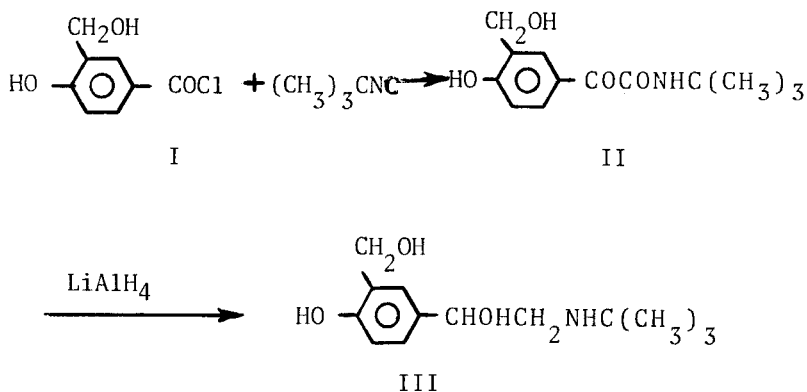
reduced with LiAlH₄ in THF under nitrogen to yield III which is subsequently debenzylated with hydrogen in the presence of Pd/C catalyst to give salbutamol IV.

- b) In 1973, Lunts and Toon (11) described another method for the preparation of several 1-phenyl-2-aminoethanol derivatives including salbutamol through reduction of methyl-5-(2-amino-1-hydroxyethyl)-salicylate I with LiAlH₄ to give the corresponding alcohol II which was then converted to salbutamol III by the reaction with tertiary butyl chloride as shown in Scheme 2.



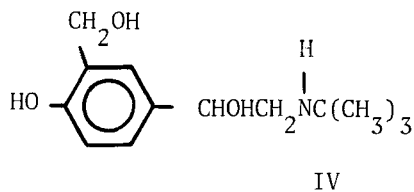
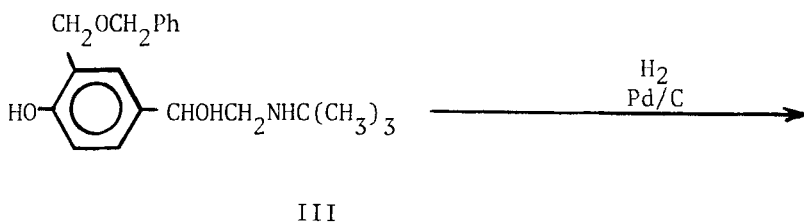
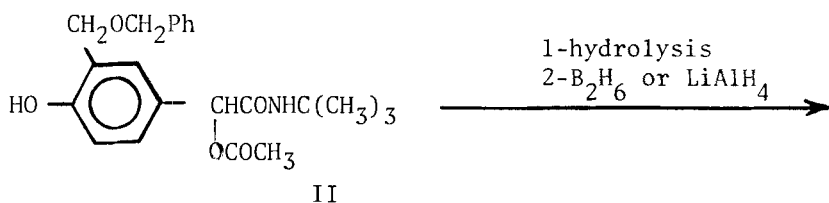
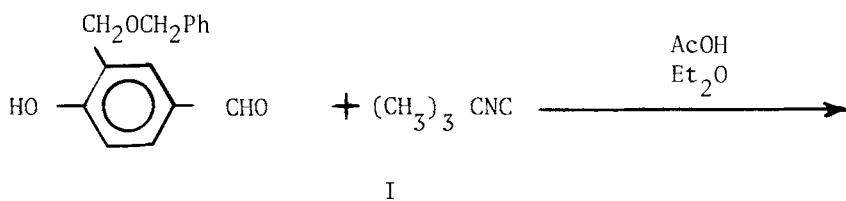
Scheme 2.

- c) Okumura *et al* (12) have prepared salbutamol through the reaction of 4-hydroxy-3-hydroxy methyl benzoyl chloride I with tertiary butyl isonitrile in benzene to give II which is reduced with LiAlH_4 to give salbutamol III as shown in Scheme 3.



Scheme 3.

- d) Kyotani *et al* (13) have prepared several 1-(-3-hydroxy methyl-4-hydroxyphenyl)-2-alkylaminoethanol derivatives including salbutamol as shown in Scheme 4.



Scheme 4.

Treating the O-protected benzaldehyde I with tertiary butyl isonitrile in the presence of acetic acid to give II. Compound II is hydrolysed followed by reduction with diborane or LiAlH_4 to give III. The latter was then subjected to hydrogenolysis with Pd/C to give salbutamol IV in about 49% yield.

4. Metabolism, Absorption and Excretion

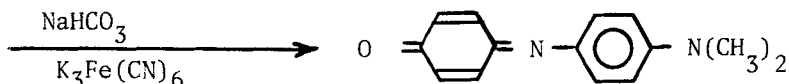
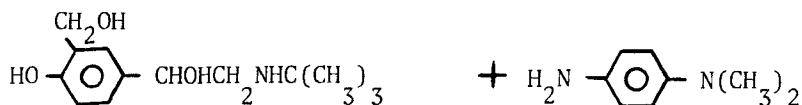
Salbutamol is readily absorbed from the gastrointestinal tract (14). Its effect occurs within 15 minutes and lasts for about 14 hours. When give by inhalation, its effect occurs within 5 minutes (14, 15). The drug is excreted in urine in about 24 hours, 50% of the dose administered by mouth or 30% of the dose by inhalation is excreted within 4 hours (15). About 80% of the tritium-labelled salbutamol given orally, intravenously or by aerosol is excreted in urine within three days. The peak-plasma concentration of salbutamol and its metabolites is $5.1\text{--}11.7\text{ }\mu\text{g\%}$ at 2.5 - 3 hours after an oral dose (4 mg of salbutamol)(7).

Evans et al (7) reported that salbutamol was extensively metabolized to a plar metabolite in humans, which possessed spectral and chemical properties different from the parent drug. The paper chromatographic properties of salbutamol, its glucuronide and the polar metabolite will be discussed later (see section 5.31). The metabolite is a conjugate which was not hydrolysed by β -glucuronidase, sulphatase, ketodase of β -glucosidase. Thus the authors reported that the metabolite formed in man was different from that formed in rat and rabbit, i.e., the metabolite is not a conjugate of glucuronic acid, sulphate or glucose.

In dog's urine, 70-90% of the drug was excreted, 10% as its glucuronide metabolite (16), while in the rabbit and rat 90 and 40% respectively was changed to the O-glucuronide. The latter possesses neither a β -stimulant or a β -blocking activity (14).

Salbutamol does not cross the blood-brain barrier to a significant extent, but it corsses the placenta barrier. In man, about 25% of an administered dose is metabolised to the 4-O-sulphate ester (17) which is contrary to Evans et al's finding (7)

The metabolic pathway of salbutamol in different species is shown in Scheme 5.



5.3. CHROMATOGRAPHIC METHODS

5.31 Paper Chromatography

Clarke (6) described a solvent system used for the paper chromatography of salbutamol consisting of citric acid: H₂O : n-butanol (4.8 gm: 130 ml: 870 ml). The drug can be detected under ultra-violet or by using potassium permanganate spray.

Evans et al (7) reported several solvent systems for the separation of salbutamol and its metabolites as shown in Table 2. Descending paper chromatography on Whatman 3mm paper [4x 55 cm] were used.

The R_f values of salbutamol and metabolites were determined by radiochromatogram scanning since H³-salbutamol was used in Evans et al's studies.

5.32 Thin Layer Chromatography

A thin layer chromatographic procedure for salbutamol has been reported (6), the solvent system consists of strong ammonia solution: methanol (1.5 : 100) which should be changed after two runs. Several visualizing agents can be used, e.g. potassium permanganate, iodine/CCl₄, Dragendorff spray, p-dimethylaminobenzaldehyde spray as well as ultraviolet light.

Table (2)

Solvent system.	R_f Values		
	Salbutamol	Salbutamol glucuronide	Polar Metabolite in man
1. n-Butanol : Acetic acid : H_2O 4 1 1.6	0.74	0.30	0.47
2. Isopropanol : Ammonia (Sp. gr. 0.88) 7 3	0.87	0.60	0.80
3. n-Butanol : Ammonia (Sp.gr.0.88): H_2O 10 1 1	0.74	0.00	0.38
4. Methanol : n-Butanol : Benzene : H_2O 4 3 2 1	0.75	0.20	0.75
5. Phenol : H_2O 4 1	0.90	0.81	0.74
6. 80% Aq.ethanol.	0.82	0.00	0.78
7. n-Butanol : Ethanol : H_2O 9 4 7	0.62	0.19	0.58

5.33 Gas Chromatography

Martin et al (20) quantitatively determined salbutamol in plasma as either its trimethylsilyl or tertiary butyl dimethylsilyl ether. The derivatives were introduced for GLC at 250° on a glass column, 1m x 4mm packed with 3% OV 101 on gas chromatograph (100-120 mesh).

5.4 Mass Fragmentography

Salbutamol among other substances were quantitatively determined by mass fragmentography after gas chromatography on coated capillaries. This has been achieved with a magnetic sector-type mass spectrometer with a closed loop control of the magnetic field and a digitally controlled high voltage supply. The method can detect picogram and nanogram amounts (21).

Martin et al (20) have developed two methods for the determination of salbutamol in human plasma using the stable isotope multiple ion recording technique. The first method involved the extraction of salbutamol from plasma as its tetraphenylboron ion pair, separated from plasma cholesterol and derivatized at its trimethylsilyl ether. The drug was determined by mass spectrometry using to measure the intensity of the fragment m/e 369. Trideuterosalbutamol was used as an internal standard. The second method involved ion-pair extraction of salbutamol into heptan-3-one. The drug was derivatized to its tertiary butyl dimethylsilyl ether and determined by GC/MS using the fragment m/e 495 and 498 (for trideuterosalbutamol tert-butyl dimethylsilyl ether derivatives).

The latter method is reported to be rapid and did not require separation of cholesterol.

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SUCCINYLBCHOLINE CHLORIDE

Penelope R. B. Foss and Steven A. Benezra

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1. Description

1.1 Name, Formula, Molecular Weight

Succinylcholine chloride

2,2'-[(1,4-dioxo-1,4-butanediyl)bis(oxy)]bis[N,N,N-trimethylethanaminium] dichloride;

bis[2-dimethylaminoethyl]succinate bis[methochloride];

2-dimethylaminoethyl succinate dimethochloride;

diacetylcholine dichloride;

suxamethonium chloride;

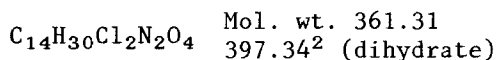
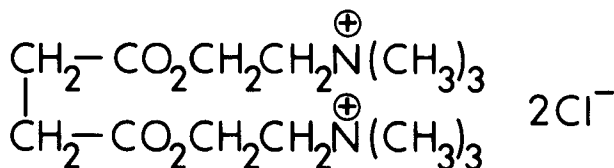
choline succinate dichloride;

succinic acid bis[β-dimethylaminoethyl] ester dimethochloride;

choline chloride succinate (2:1);

Listenon; Anectine chloride; Scoline chloride; Lysthenon; Midarine;

Quelicin chloride; Sucostrin chloride; Ultrapal chloride; Succicuran¹



Succinylcholine chloride exists as a dihydrate at room temperature. All data presented here is for the dihydrate form unless otherwise stated.

1.2 Appearance, Color, Odor

Succinylcholine chloride is characterized as having white, odorless, crystals.³

2. Physical Properties

2.1 Infrared Spectrum

The infrared spectrum of succinylcholine chloride is shown in Figure 1. It was taken as a 0.2% dispersion of succinylcholine chloride in KBr with a Nicolet Model 7199 FT-IR spectrophotometer. Table I gives the infrared assignments consistent with the structure of succinylcholine chloride.⁴

Table I

Infrared Spectral Assignments for Succinylcholine Chloride

<u>Band (cm⁻¹)</u>	<u>Assignment</u>
1151,1048	C-O-C stretch (ester)
1738	C=O stretch (ester)
1312	CH ₂ wagging vibration
1428	C-H bending vibration from CH ₃
1481	C-H bending vibration from CH ₂ and CH ₃
2930-3023	C-H stretch
2957	C-H stretch from CH ₃
2930	C-H stretch from CH ₂
3426	overtone of C=O stretch
3484	O-H stretch from water of hydration

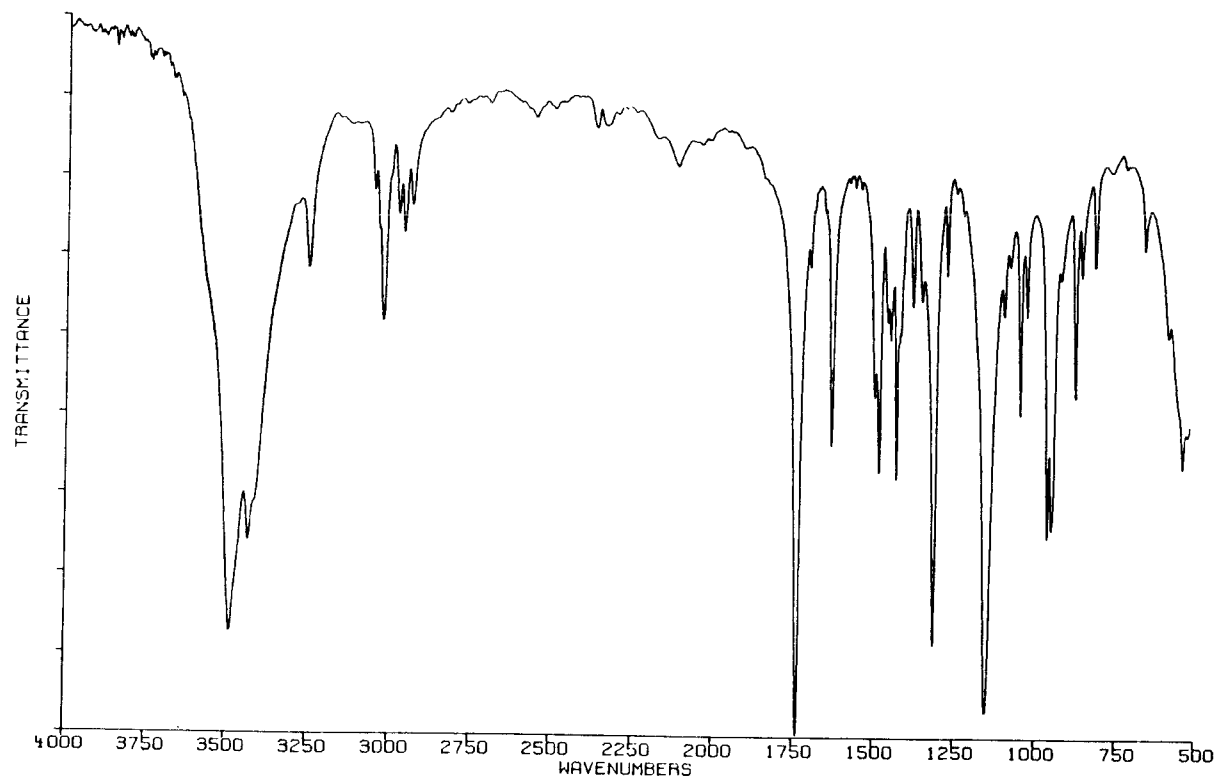


Figure 1 Infrared Spectrum of Succinylcholine Chloride

2.2 Nuclear Magnetic Resonance (NMR) Spectrum

2.21 Proton NMR Spectrum

The proton NMR spectrum of succinylcholine chloride is shown in Figure 2. It was obtained with a Varian CFT-20 80 MHz spectrometer. Deuterated water was used as the solvent with tetramethylsilane as an internal standard. Based on the NMR spectrum, the following proton assignments are made.⁵

<u>Proton</u>	<u>Chemical Shift (ppm)</u>
$\begin{array}{c} \text{O} \\ \parallel \\ \text{CH}_2\text{C}-\text{O} \end{array}$	2.69 singlet
$(\text{CH}_3)\text{N}^+$	3.13 singlet
CH_2N^+	3.65 multiplet
$\begin{array}{c} \text{O} \\ \parallel \\ \text{CH}_2\text{OC} \end{array}$	4.50 multiplet

2.22 Carbon-13 NMR Spectrum

The carbon-13 NMR spectrum of succinylcholine chloride is shown in Figure 3. It was obtained with a Varian XL-100 25 MHz spectrometer equipped with a Nicolet data system. Deuterated water was used as the solvent with tetramethylsilane as an internal standard. Based on the NMR spectrum, the following carbon assignments are made.⁵

<u>Carbon</u>	<u>Chemical Shift (ppm)</u>
$\text{C}=\text{O}$	176.36 singlet
CH_2N^+	67.19 triplet
CH_2O	61.26 singlet
CH_3N^+	56.52 triplet
$\begin{array}{c} \text{CH}_2 \\ \\ \text{CH}_2 \end{array}$	31.25 singlet

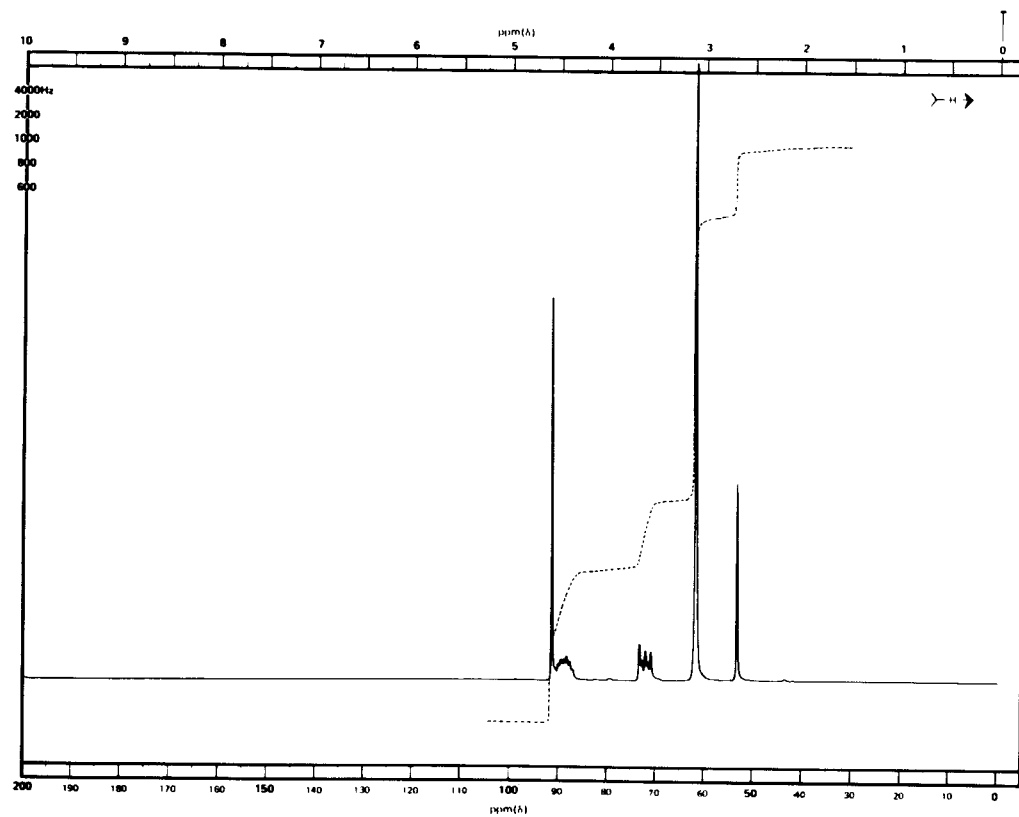


Figure 2 Proton NMR Spectrum of Succinylcholine Chloride

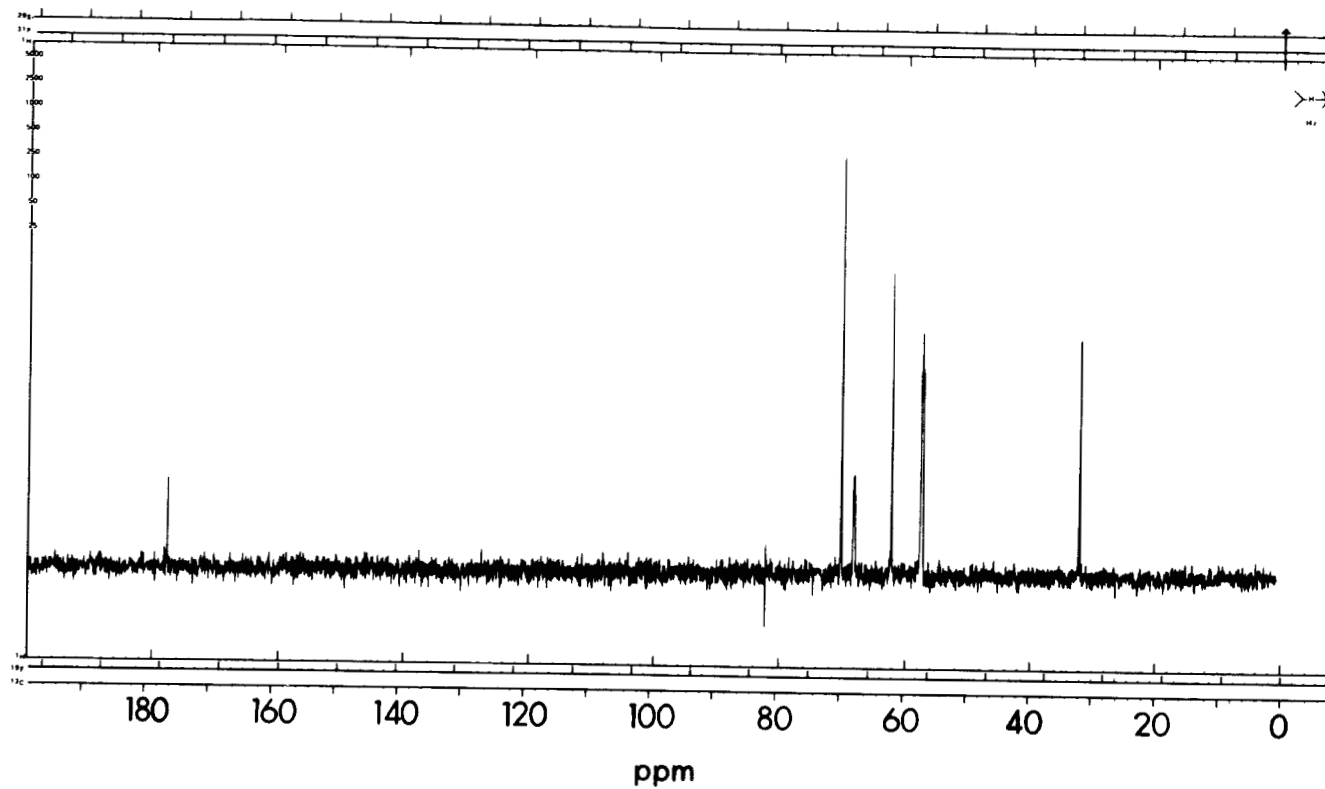


Figure 3 Carbon-13 NMR Spectrum of Succinylcholine Chloride

2.3 Ultraviolet (UV) Spectrum

Succinylcholine chloride does not absorb UV radiation above 220 nm.

2.4 Mass Spectrum

The low resolution field desorption mass spectrum of succinylcholine chloride is shown in Figure 4. It was obtained with a Varian MAT 731 mass spectrometer coupled to a VG multispec data system. The spectrum was recorded at 20 mA. The assignment of fragment ions is given below.⁶

m/z 78	molecular ion of dimethyl sulfoxide used as a chemical marker
m/z 325	$[M-Cl]^+$
m/z 275	thermal elimination of CH_3Cl followed by loss of Cl^- and acceleration of the resultant quaternary ammonium ion $[M-CH_3Cl-Cl]^+$
m/z 585	result of two molecules (after thermal elimination of CH_3Cl from each molecule) clustering and losing Cl^- $[2M-2(CH_3Cl)Cl]^+$

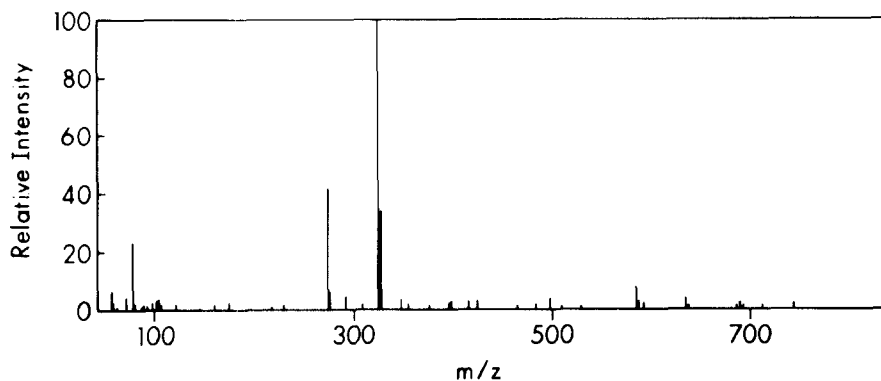


Figure 4 Mass Spectrum of Succinylcholine Chloride

2.5 Melting Point¹

Succinylcholine chloride dihydrate crystals melt at 156-163°C. The anhydrous form melts at about 190°C.

2.6 Solubility¹

Succinylcholine chloride is freely soluble in water (1 g/mL), soluble in 95% ethanol (0.42 g/100 mL). It is sparingly soluble in benzene, chloroform and practically insoluble in ether.

3. Synthesis

Figure 5 outlines a synthesis of succinylcholine chloride. Diethyl succinate (1) is condensed with dimethylethanolamine (2) to yield dimethylaminoethylsuccinate (3). The dimethylaminoethylsuccinate is quaternized with methyl chloride (4) to give succinylcholine chloride (5).^{7a,b}

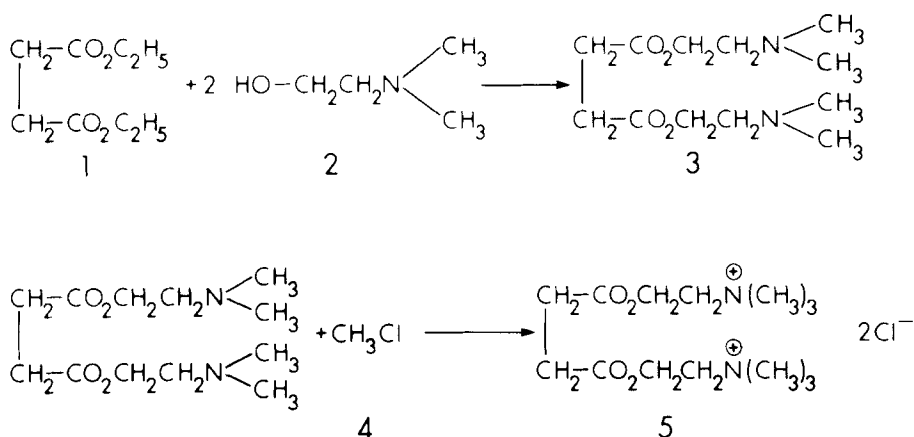


Figure 5 Synthesis of Succinylcholine Chloride

4. Stability³

Succinylcholine chloride is stable in crystalline form. Aqueous solutions hydrolyze at an increasing rate with the increase of pH, temperature, and concentration. A five percent solution at room temperature shows very slow hydrolysis at pH 3-5, moderately fast hydrolysis at pH 7.4 and very rapid hydrolysis at pH 10-11. A five percent solution at pH 3-5 maintains potency for more than two years when kept at 4°C, and rapidly loses potency at 100°C.

5. Methods of Analysis

5.1 Elemental Analysis¹ (Anhydrous)

Element	C	H	Cl	N	O
% calculated	46.54	8.37	19.63	7.75	17.71

5.2 Titration Analysis

5.21 Succinylcholine chloride is dissolved in a mixture of glacial acetic acid and mercuric acetate. The solution is titrated with 0.1N perchloric acid with crystal violet as the indicator. Each mL of 0.1N perchloric acid is equivalent to 18.07 mg of $C_{14}H_{30}Cl_2N_2O_4$.²

5.22 An injection sample of succinylcholine chloride is diluted with water and extracted with ether. The collected ether extracts are washed with water and discarded. The combined water washings are extracted with ether, added to the aqueous phase from the initial extraction, and heated on a steam bath until just free of ether odor. The solution is neutralized with 0.1N sodium hydroxide using bromothymol blue as the indicator. Twenty-five milliliters of 0.1N sodium hydroxide are added to the sample solution and the blank, then refluxed for forty minutes. The cooled samples are titrated with 0.1N HCl. Each mL of 0.1N NaOH is equivalent to 18.07 mg of $C_{14}H_{30}Cl_2N_2O_4$.²

5.23 Two milliliters of a succinylcholine chloride injection sample are transferred to a 150-mL flask. Sodium thiosulfate (0.005N) and KIO_3/KI reagent are added to the flask. The flask is allowed to stand for ten minutes. A mucilage of starch is added and

the excess sodium thiosulfate is titrated with 0.005N iodine. The result of the blank is subtracted, and the amount of succinic acid generated by hydrolysis of the succinylcholine chloride in aqueous solution is calculated.⁸

5.3 Chromatography

5.31 High Performance Liquid Chromatography

High performance liquid chromatography has been used to determine succinylcholine chloride in injection and Flo-pack® samples.⁹ A stainless steel column (25 cm x 3.9 mm i.d.), packed with Partisil® 10 was used for the separation. The mobile phase, methanol/tetramethylammoniumchloride (9:1), was adjusted to pH 3 with HCl. The UV detector was set to 214 nm. The flow rate was ~0.75 mL/min which gave a seven-minute retention time for succinylcholine chloride.

5.32 Paper Chromatography

Paper chromatography⁸ was used for the determination of succinylcholine chloride in injection samples. The paper was Whatman No. 1 and the mobile phase was n-propanol/benzyl alcohol/water (5:2:2). The development time was 18 hr. After the chromatogram was air dried, the separated sample components were visualized with 0.05% iodine solution. The observed R_f value for choline was 0.25, and for succinylcholine was 0.105.

A second mobile phase⁸ used for paper chromatography was n-butanol/acetic acid/water (4:1:5). The sample components were visualized with Dragendorff's reagent. Choline gave a violet spot and succinylcholine gave an orange spot.

5.33 Thin Layer Chromatography

Thin layer chromatography¹⁰ was used for the detection of succinylcholine chloride. The thin layer plate was a Merck silica G-60 with no fluorescent indicator. The mobile phase was acetone/1.0N HCl (1:1). The sample was dissolved in water to a concentration of 1 mg/mL and spotted in 1 μ L portions. After the developed plate was air dried, the succinylcholine

chloride was visualized with Dragendorff's reagent. Succinylcholine chloride had an R_f of 0.47.

5.4 Biological Tests

5.41 Rat Respiration Test¹¹

A polyethylene tube was passed into the trachea of an anaesthetized rat. The rat's respiration amplitude was measured by the amount of water displaced in a water-filled glass flask connected to the endotracheal tube and an oxygen source. The half-height of the maximum amplitude was recorded. Succinylcholine chloride was administered and respiration depression ensued. The time, elapsing from the moment of injection of the drug until the resumed respiration raised the level of the manometer to the previously marked half-height, was measured and recorded as the response to a given dose. By linear regression, a curve was drawn of the time of respiratory depression versus the log of the dose of succinylcholine chloride.

5.42 Frog Muscle Response¹²

The amount of succinylcholine chloride in either blood serum or urine samples was determined by adding a sample to a Ringer solution (NaCl 0.6%, KCl 0.042%, CaCl₂ 0.032%, NaHCO₃ 0.05%, dextrose 0.05%) surrounding a frog muscle. The contraction of the muscle displaced a recorder pen which traced a peak on chart paper. The height of the peak was proportional to the dose level of succinylcholine. A dose response curve was drawn and the concentration of an unknown succinylcholine solution was determined.

6. Metabolism and Pharmacokinetics

6.1 Metabolism

Succinylcholine chloride, a short-acting depolarizing muscular relaxant, is a positively charged small linear molecule consisting essentially of two acetylcholine molecules.¹³ Its low toxicity is due to the relatively inert nature of the drug and its rapid breakdown to succinic acid and choline.³

Succinylcholine chloride hydrolysis in serum occurs in a two-stage reaction: one, the formation of

succinylmonocholine and choline, and two, the further breakdown of succinylmonocholine to choline and succinic acid. The first step³ is six times faster than the second. The two enzymes in the body concerned with the breakdown of choline esters are acetylcholinesterase and cholinesterase. Acetylcholinesterase is present in the neuromuscular junction, nervous tissue, and in the red blood cells. It does not hydrolyze succinylcholine to an appreciable extent. Cholinesterase is present in most tissues in the body: plasma, nervous tissue, cardiac muscle, intestine, and skin.

Up to 90%³ (80%¹⁴) of succinylcholine is hydrolyzed by plasma cholinesterase before it reaches the neuromuscular junction. Ten percent³ of succinylcholine is excreted unmetabolized in the urine.

6.2 Tissue Distribution

When succinylcholine is administered intravenously there is a rapid initial distribution of the drug throughout the extracellular fluid volume to the muscle mass and neuromuscular junction.¹⁴

Whole body distribution of succinylcholine was determined in a study involving near-term macaca mulatta monkeys.¹⁵ The radio-labelled drug was injected into the umbilical vein of developing feti. The feti were delivered by cesarean section and examined for the whole body distribution of ¹⁴C succinyldicholine hydroiodide.

The highest concentration of radioactivity was found in the intravascular space, kidney, and other highly perfused organs and cartilage. A lower concentration was found in the lung, skeletal muscle, and bone marrow. Radioactivity was absent in the brain, vitreous body, and cerebrospinal fluid. The activity in the skin, primary dentine, and in the blood vessels, and intestinal walls was especially marked. Radioactivity was rapidly taken up in the sclera, choroid layer, iris, ciliary body, and extraocular muscle.

6.3 Pharmacokinetics

In an *in vitro*¹² study, it was found that 85% of succinylcholine is destroyed within the first half

minute following the mixing with blood. After two minutes only 5% of the drug remained.

The neuromuscular blocking effect is terminated by redistribution of succinylcholine away from the neuromuscular junction and only to a limited extent by further hydrolysis in the plasma by plasma cholinesterase (pseudocholinesterase).¹⁴

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TRIOXSALEN

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and
Mohammed A. Loutfy

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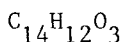
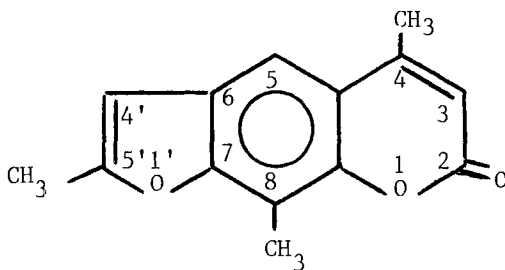
- a- 2,5,9-Trimethyl-7H-furo(3,2-g)benzopyran-7-one
- b- 2,5,9-Trimethyl-7H-furo(3,2-g)[1] benzo-pyran-7-one
- c- 6-Hydroxy- β ,2,7-trimethyl-5-benzofuran-acrylic acid δ -lactone
- d- 7H-Furo [3,2-g][1] benzopyran-7-one, 2,5,9-trimethyl
- e- 4,5',8-Trimethylpsoralen.

1.12 Generic Names

4,5,8-Trimethylpsoralen; Trimethylpsoralen; Trioxsalen; Trioxysalen; TMP.

1.13 Trade Names

Trisoralen ; Trioxsalen.

1.2 Formulae1.21 Empirical1.22 Structural1.3 Molecular Weight

228.25 (1,2) ; 228.24 (3) ; 228.2 (4).

1.4 Elemental Composition

C, 73.67% ; H, 5.30% ; O, 21.03%.

1.5 Appearance, Color, Taste, Odor

Crystalline solid or prisms, white to off-white or grayish, tasteless, odorless.

2. Physical Properties

2.1 Melting Point

Trioxsalen melts at about 230° (1,2,4) or at 234.5 - 235 (3).

2.2 Solubility

Practically insoluble in water, slightly soluble in alcohol (1g in 1150 ml), sparingly soluble in chloroform (1g in 84 ml), fairly soluble in methylenedichloride (1gm in 43 ml).

2.3 Identification

- i) The infrared absorption spectrum of a mineral oil dispersion of trioxsalen, previously dried at 105° for 6 hours, exhibits maxima only at the same wavelengths as that of a similar preparation of USP Trioxsalen Reference Standard (2).
- ii) The ultraviolet absorption spectrum of a 1 in 200,000 solution in chloroform exhibits maxima and minima at the same wavelengths as that of a similar preparation of USP Trioxsalen Reference Standard, concomitantly measured (2).

2.4 Spectral Properties

2.41 Ultraviolet Spectrum

The UV spectrum of trioxsalen in methanol was scanned using Pye Unicam SP 800 spectrophotometer; from 400 - 200 nm. Three maxima and three minima were observed:

λ_{\max}	$\text{Log } \epsilon$	λ_{\min}	$\text{Log } \epsilon$
248	4.35	225	4.09
296	3.99	272	3.68
338	3.80	320	3.79

The spectrum is shown in Figure 1. Other UV spectral data have been also reported (3,5,6).

2.42 Infrared Spectrum

The IR spectrum of trioxsalen is recorded as a nujol mull on a Unicam SP 1025 spectrophotometer and is shown in Figure 2. The assignments for the characteristic bands in the infrared spectrum are listed in Table 1.

Table 1

Frequency Cm^{-1}	Assignment
3120	CH_3
1710	C=O (α -pyrone)
1640	C=C (α -pyrone)
1620	
1600	C=C (aromatic)
1190	
1170	C-O-C (α -pyrone;furan)
1110	
880	Furan ring
840	
810	Isolated H (Penta substituted benzene)

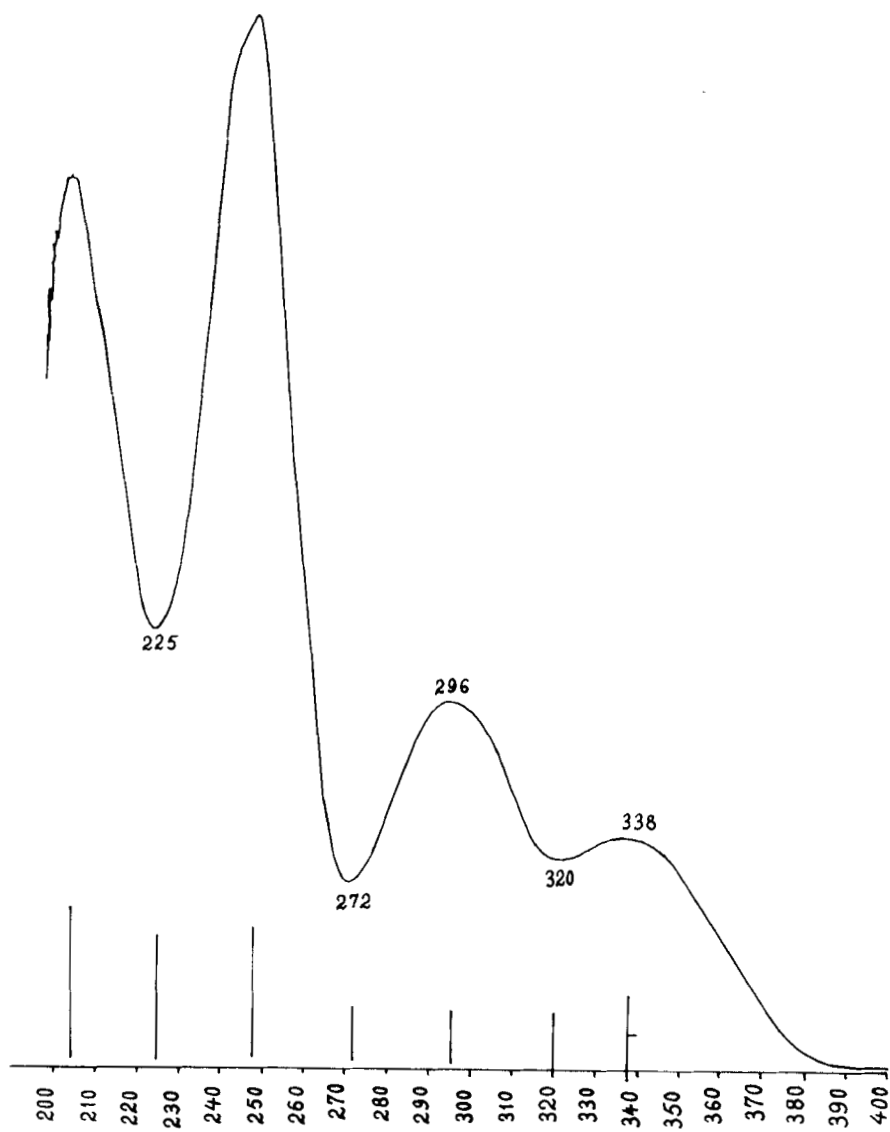


Fig. 1. UV spectrum of Trioxsalen in Methanol.

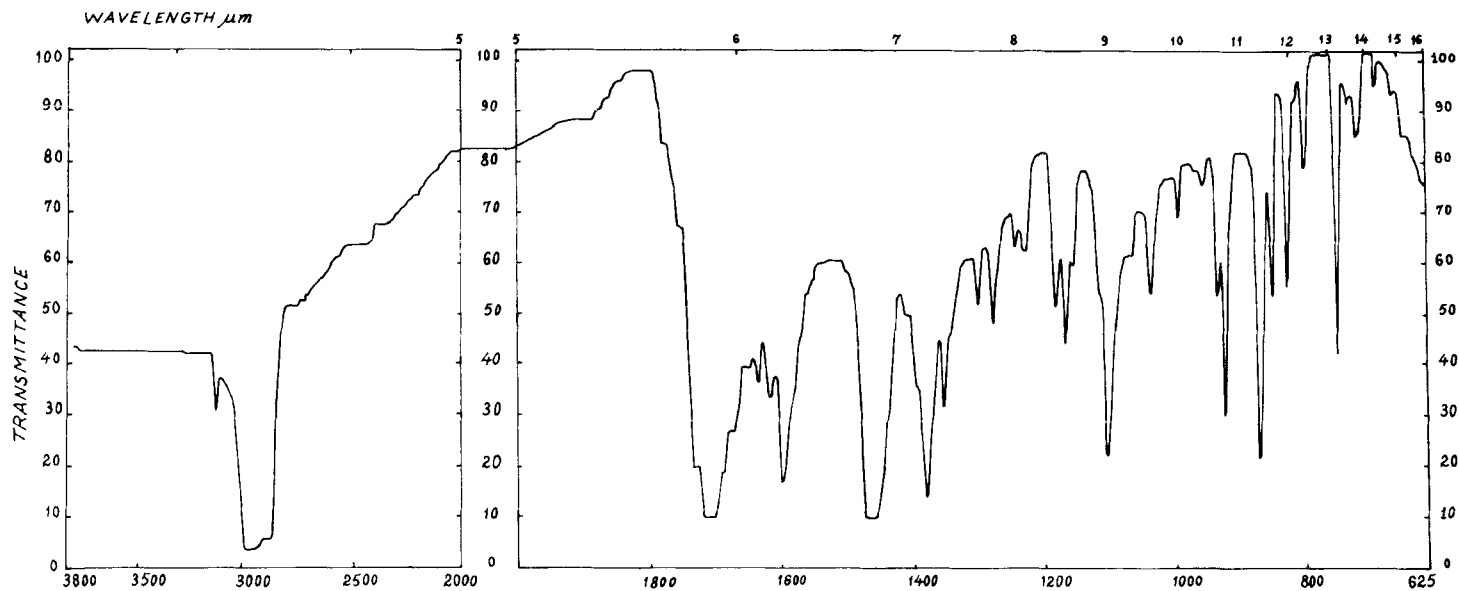


Fig. 2. IR spectrum of Trioxsalen in nujol.

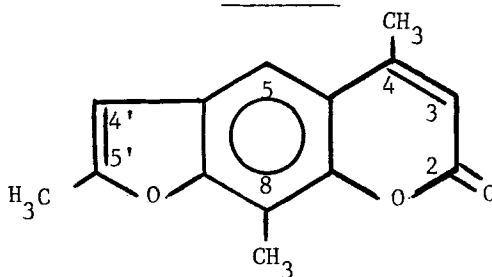
Other finger print bands characteristic of trioxsalen are : 1465, 1385, 1360, 1305, 1280, 1250, 1235, 1045, 1000, 945, 930, 860 and 760 (5-7).

2.43 Nuclear Magnetic Resonance Spectrum

2.431 Proton Spectrum

The proton magnetic resonance spectra of trioxsalen and other psoralens have been reported (8,9). A typical PMR spectrum of trioxsalen is shown in Figure 3. The sample was dissolved in deuterated chloroform and the spectrum was recorded and the spectrum was recorded on a Varian XL-200, 200 MHz NMR spectrometer using tetramethylsilane as a reference standard. The PMR spectral assignments of trioxsalen are given in Table 2.

Table 2: PMR Characteristics of Trioxsalen
in CDCl₃



Chemical shifts (δ)					
4-CH ₃ d	8-CH ₃ s	5'-CH ₃ d	3-H d	5-H s	4'-H d
2.47	2.55	2.50	6.22	7.49	6.41
2.46		2.49	6.21		6.40

S = singlet ; d = doublet

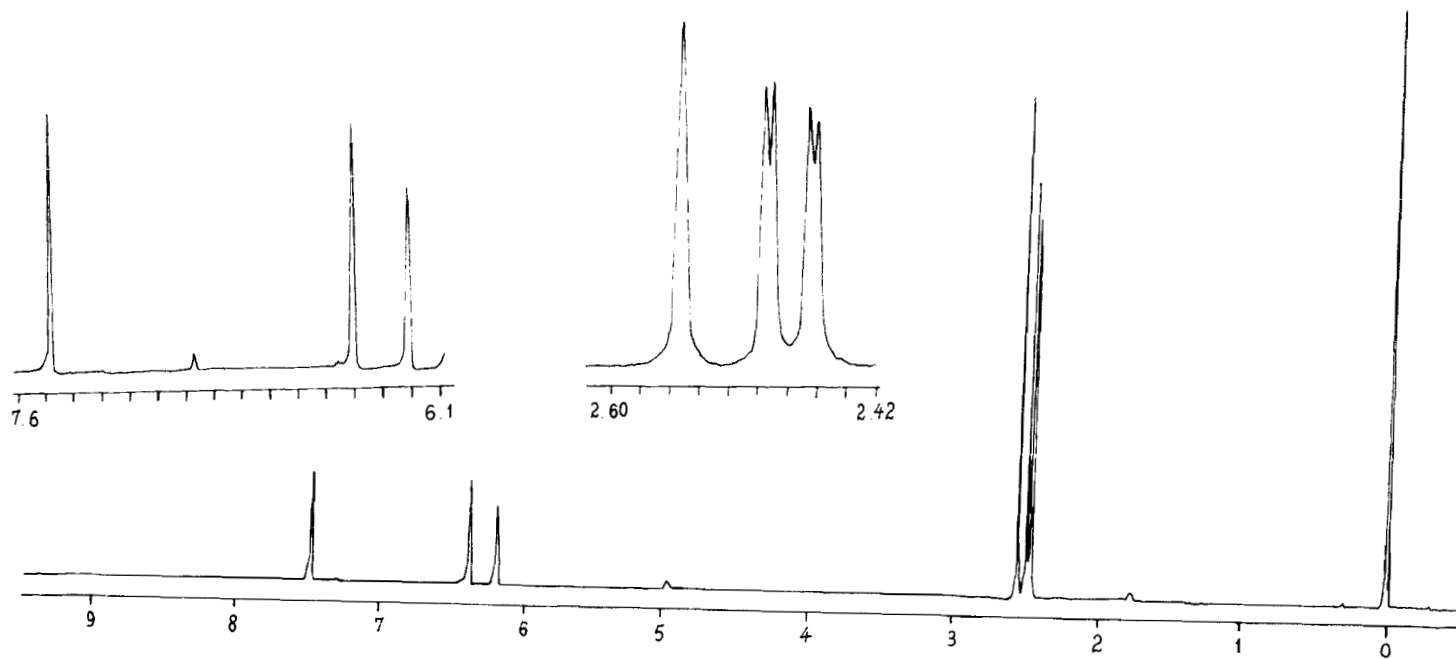
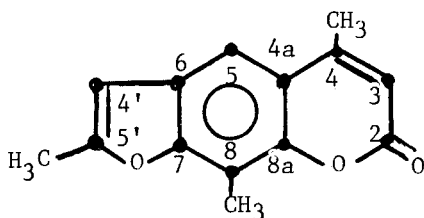


Fig. 3. PMR spectrum of Trioxsalen and Tetramethylsilane in CDCl_3 .

2.432 ^{13}C -NMR Spectrum

The fully decoupled ^{13}C -NMR spectrum of trioxsalen in deuterated chloroform is given in Figure 4. Proton-coupled (off resonance) spectrum in deuterated chloroform is also shown in Figure 5. These spectra were obtained with 50.3 MHz on a Varian XL-200 -200 MHz NMR spectrometer at ambient temperature using a broad band 10 mm probe. The sample was run at concentrations ca 1-2 M in deuterated chloroform with tetramethylsilane as an internal standard. The proton - coupled spectrum was recorded under gated-decoupling condition. The assignment of the individual signals based on the proton - decoupled spectrum is given in Table 3.

Table 3: ^{13}C -Chemical Shifts of Trioxsalen

Carbon	Chemical Shift ppm	Carbon	Chemical Shift ppm
C-2	161.5	C-8	116.0
C-3	112.7	C-8a	148.9
C-4	155.4	C-4'	157.3
C-4a	109.0	C-5'	102.6
C-5	112.1	4-CH ₃	14.2
C-6	125.4	8-CH ₃	8.5
C-7	153.2	5-CH ₃	19.2

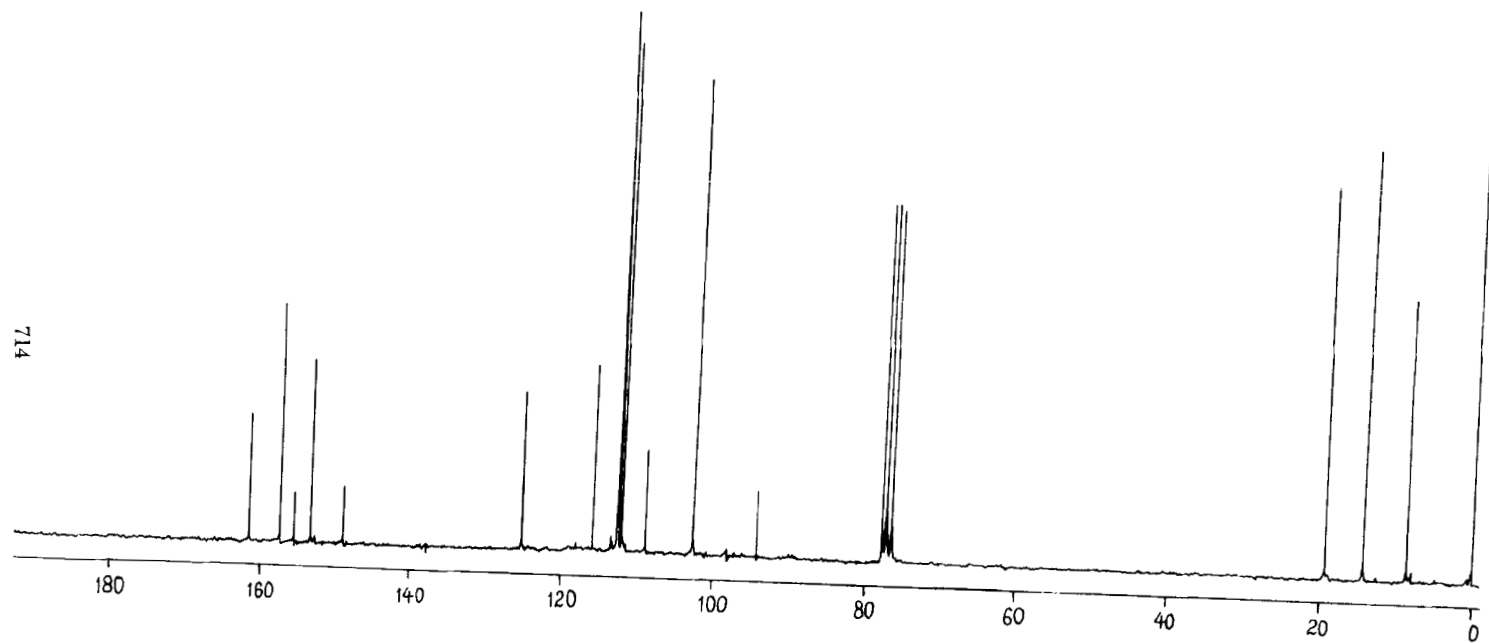


Fig. 4. ^{13}C -NMR spectrum of Trioxsalen in CDCl_3 .

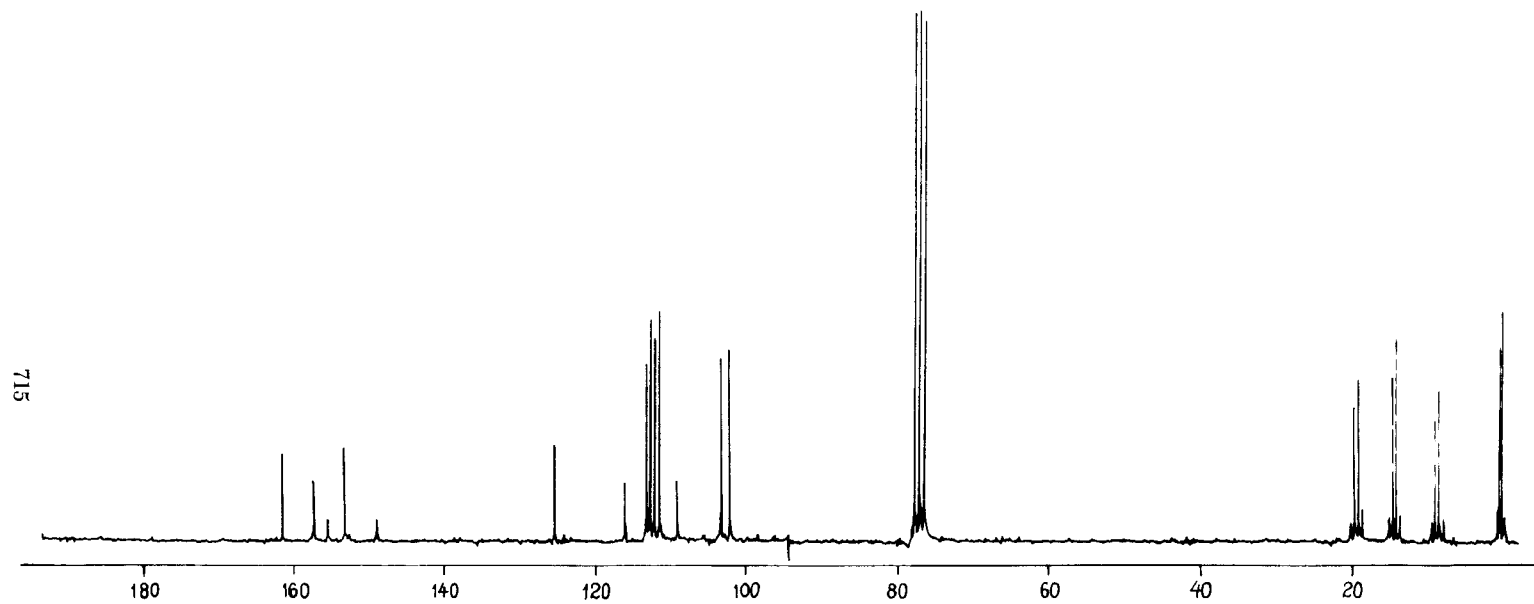


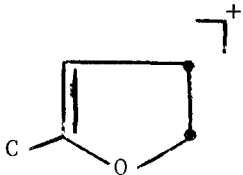
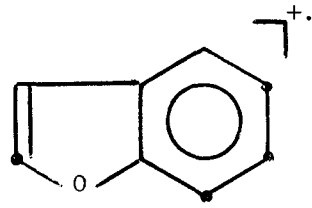
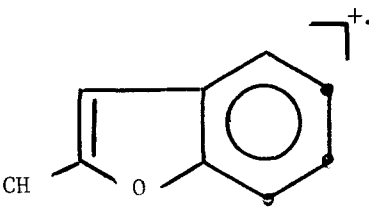
Fig. 5. ^{13}C -NMR proton-coupled spectrum of Trioxsalen in CDCl_3 .

Other ^{13}C -NMR chemical shift data of other psoralens have been also reported (10-13).

2.44 Mass Spectrum

The mass spectrum of trioxsalen obtained by conventional electron impact ionization at 70 eV, shows a molecular ion M^+ at m/e 228. The M^+ ion peak is the base peak (Figure 6). The m/e for the most prominent fragments and the corresponding ions are proposed in Table 4.

Table 4: Prominent Fragments and Corresponding Ions of Trioxsalen

m/e	Relative Intensity	Ion
77	5.3	
115	7.9	
127	4.8	

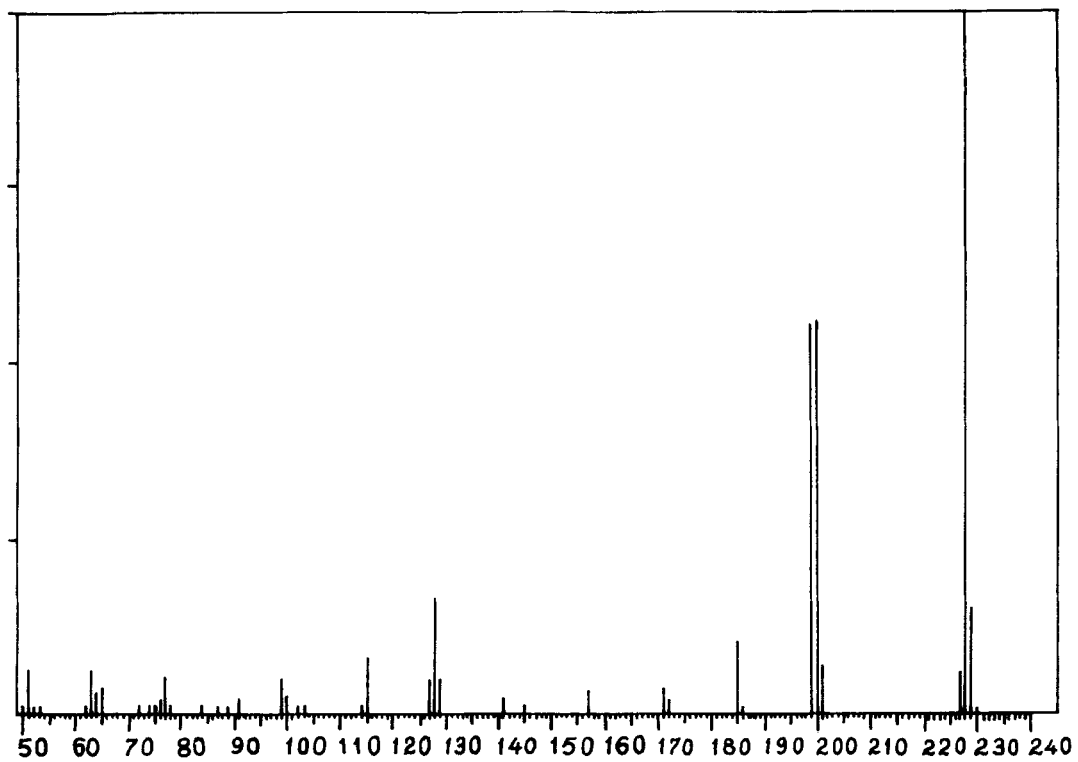
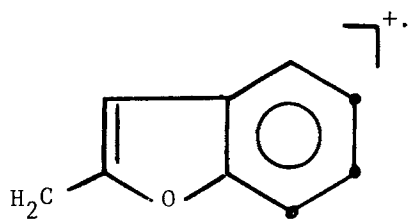


Fig. 6. Mass spectrum of Trioxsalen.

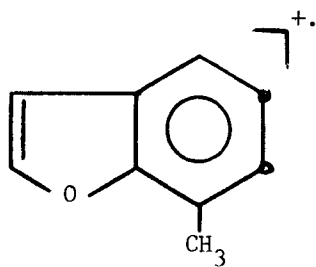
128

16.8



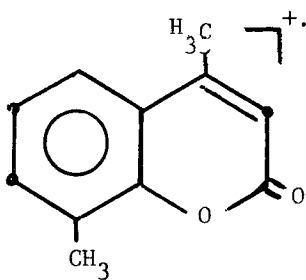
129

4.8



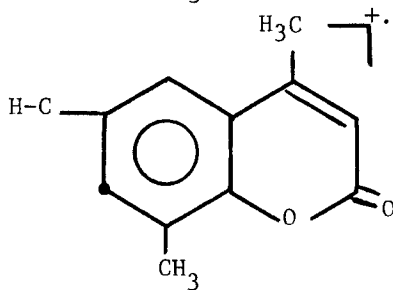
171

3.6



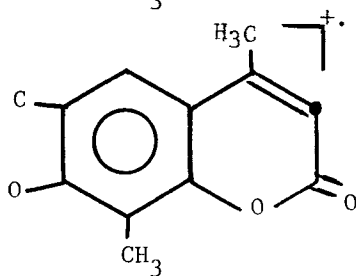
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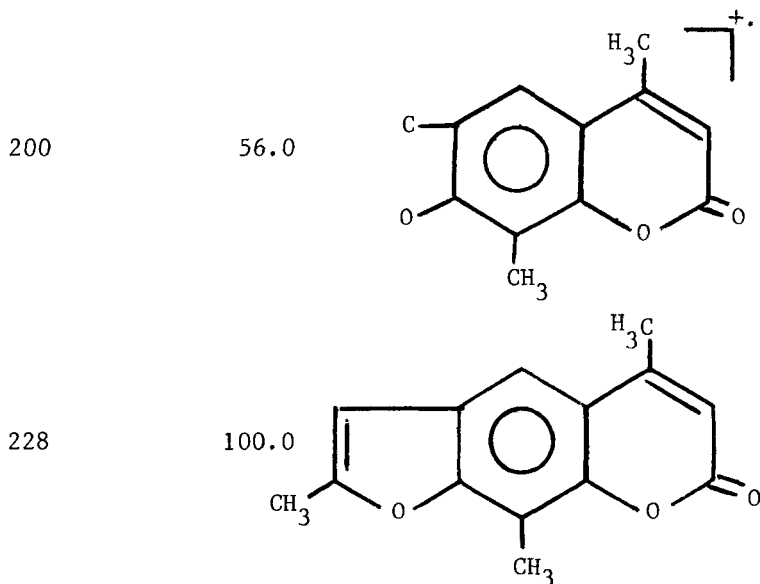
10.6



199

55.6





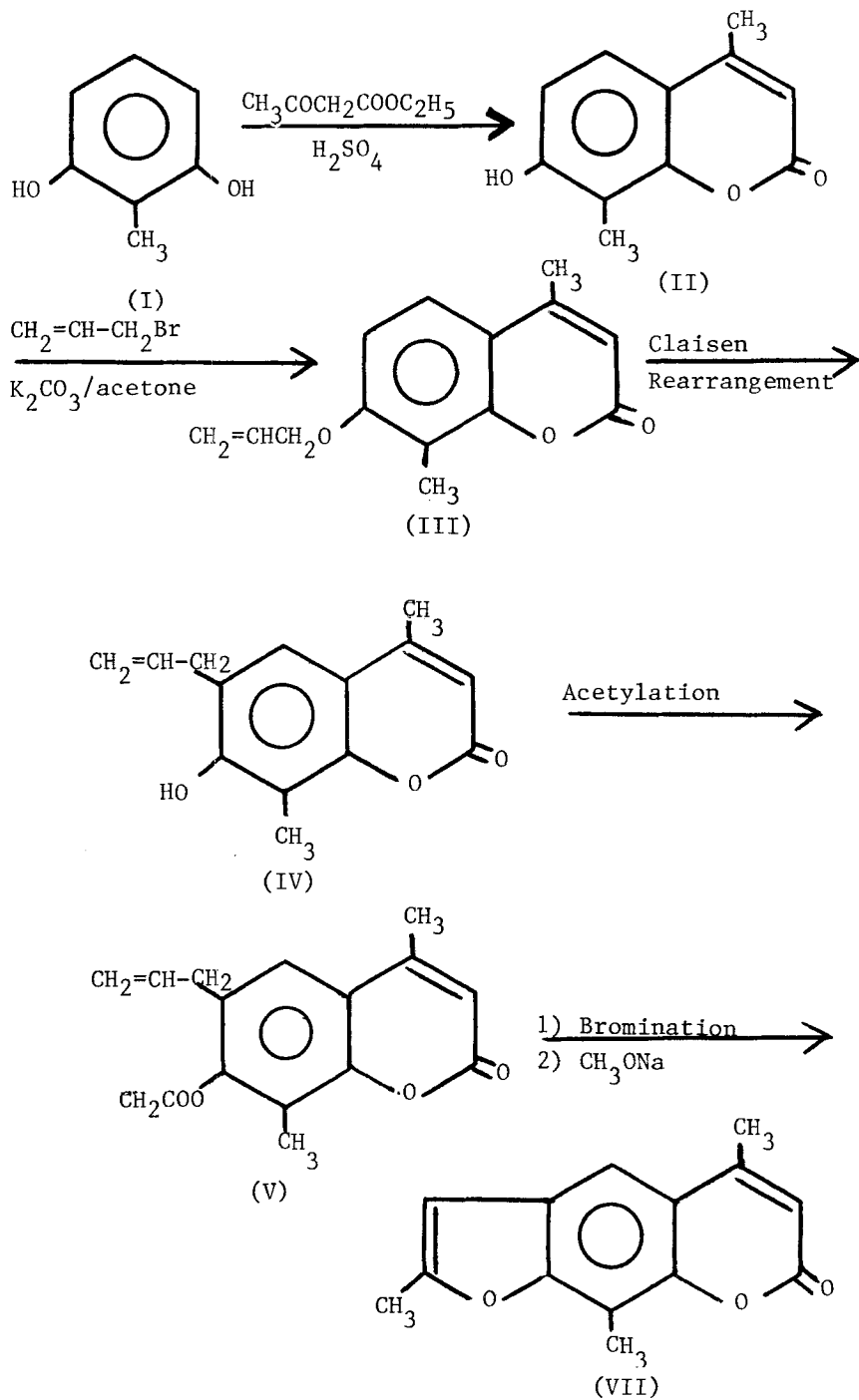
3. Isolation

The isolation of trioxsalen from celery diseased with the fungus *sclerotinia sclerotiorum* has been reported (14).

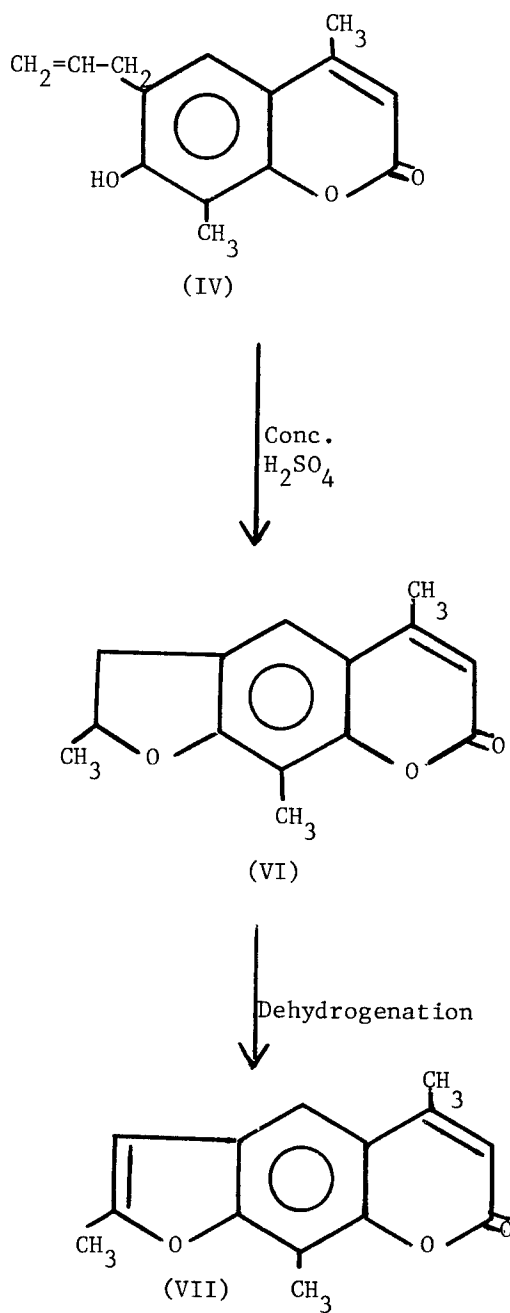
4. Synthesis

The synthesis of trioxsalen is based on the conversion of 7-allyloxy-4,8-dimethylcoumarin intermediate (III) into 6-allyl-7-hydroxy-4,8-dimethylcoumarin (IV) by Claisen rearrangement (1,6,9,15,17). This procedure involves the cyclisation of the starting material 2-methylresorcinol (I) with ethylacetoacetate, with the aid of sulfuric acid. The reaction is a Pechmann type condensation and gives 7-hydroxy-4,8-dimethylcoumarin (II). The latter is treated with allyl bromide and potassium carbonate in acetone to give 7-allyloxy-4,8-dimethylcoumarin (III) which, on reacting with acetic anhydride in the presence of *N,N*-diethylaniline and anhydrous sodium acetate, rearranges, and esterifies to give 7-acetoxy-6-allyl-4,8-dimethylcoumarin (V). Bromination of V followed by reaction with sodium methoxide yields trioxsalen (VII) (Scheme A). The 7-hydroxy group of IV is acetylated to minimise the possibility of ring bromination during the addition of one

Scheme A.



Scheme B.



equivalent of bromine to the allylic double bond.

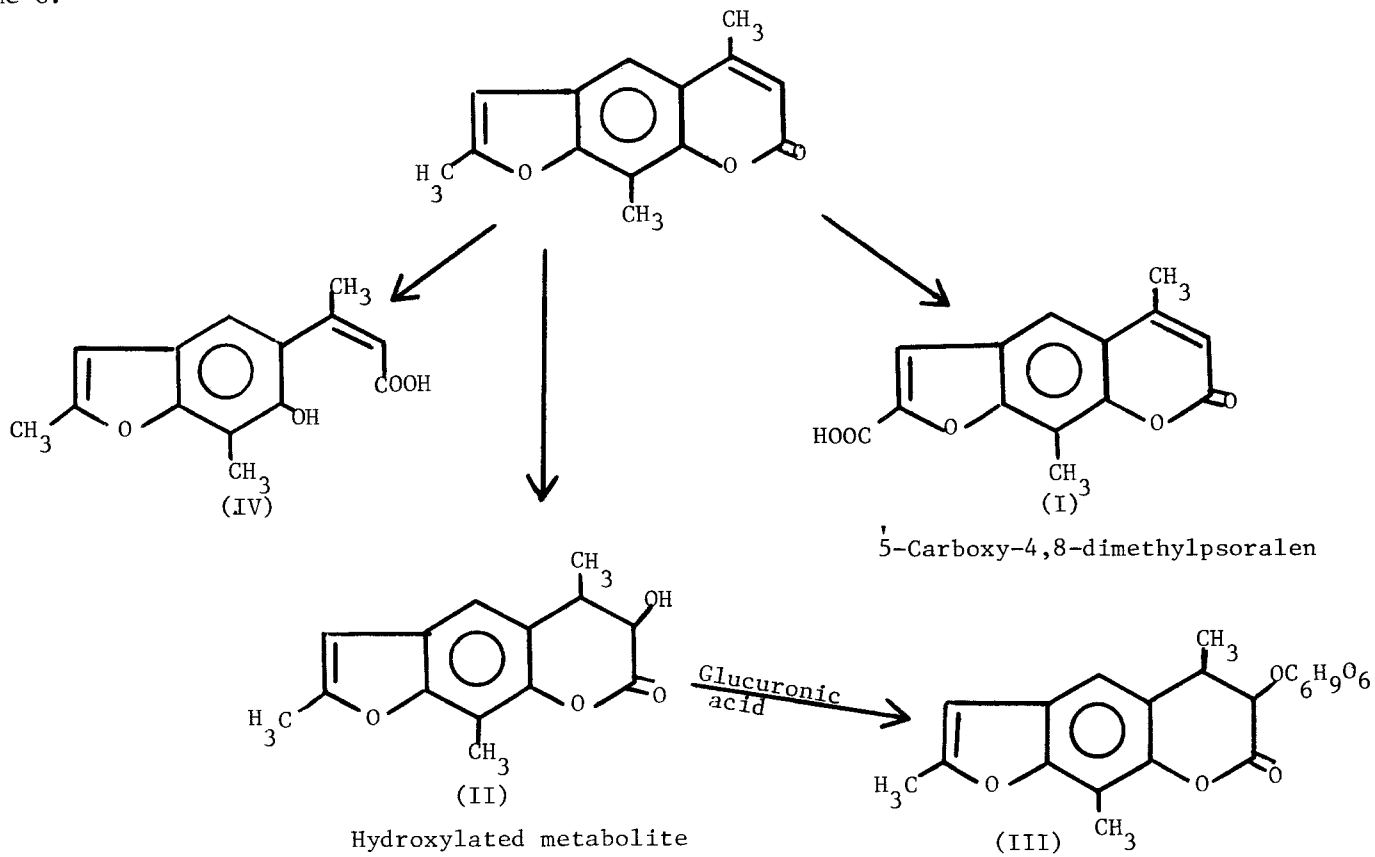
An improved synthesis of trioxsalen has been devised by Parekh and Trivedi (18). In this approach, the addition of furan ring to the 6-allyl umbelliferone intermediate (IV) has been achieved by mere trituration with concentrated sulfuric acid, at room temperature, to give 4',5'-dihydropсорalen derivative (VI). The latter, on dehydrogenation affords trioxsalen (VII) (Scheme B).

5. Metabolism

The kinetics of absorption, metabolism and excretion of trioxsalen were studied in mice and human volunteers (19). Groups of mice received ^3H -trioxsalen either orally or intraperitoneally. Urine, blood, faeces, skin and viscera were obtained at different time intervals. Trioxsalen or its metabolites were extracted and analysed for radioactivity. Over 88% of trioxsalen, after oral or intraperitoneal administration were excreted in the urine within 8 hours and over 90% within 12 hours. Distribution patterns of trioxsalen radioactivity at different time intervals in blood and various organs (liver, skin, heart, lung, brain, intestines, kidney, and spleen) revealed that trioxsalen was selectively present in liver, skin, and blood and was barely detectable in other organs. Highest values were obtained between 2 and 6 hours and diminished rapidly thereafter. Trioxsalen was metabolised in liver and excreted in urine as several distinct fluorescent metabolites, one of which appeared to be hydroxylated trioxsalen (II) (hydroxylation at 3 position). In men receiving 40 mg unlabelled trioxsalen, 80% of the administered dose was excreted in urine within 8 hours as hydroxylated or glucuronide (III) derivatives. Pathak et al (20) have reported that trioxsalen gives as a principal metabolite 5'-carboxy-4,8-dimethylpsoralen (I). This compound is inactive as skin photosensitizer and to this transformation has been attributed the difference in the photoreactivity of trioxsalen when topically applied or systematically administered (21,22).

It has been also reported (23) that one of the major metabolites of trioxsalen crystallised from urine of mice and men showed fluorescence activation and emission wave lengths of 355 and 430 nm. Infrared spectrum of trioxsalen metabolite revealed intact methyl groups, absence of OH bond at 3300 cm^{-1} and a new peak

Scheme C.



at 1225 cm^{-1} . NMR spectrum revealed opening of the lactone ring but no demethylation (IV). Therefore, metabolism of trioxsalen involves (a) hydroxylation, (b) glucuronidation, (c) opening of lactone ring with possible formation of fluorescent carboxylated moiety, and (d) oxidation of the 5'-methyl group to yield the 5'-carboxy derivative (Scheme C).

6. Photoreactions between Trioxsalen and Nucleic Acids

The photochemical addition of trioxsalen to the pyrimidine bases of DNA is considered the molecular basis for explaining its photobiological effects. Several studies (24-35) have been carried out concerning the formation of molecular complexes between trioxsalen (or other psoralens) and DNA, the mechanism of the photoaddition, the Kinetics of formation of the various photoadducts, and the specific receptor sites of DNA.

7. Methods of Analysis

7.1 Spectrophotometry

The official method adopted by the U.S.P. XIX (1975) is a spectrophotometric (2). The procedure is as follows:

Transfer about 50 mg of Trioxsalen, accurately weighed, to a 100 ml volumetric flask, add chloroform to volume, and mix. Transfer 1 ml of this solution to a 100 ml volumetric flask, add chloroform to volume, and mix. Dissolve an accurately weighed quantity of USP Trioxsalen Reference Standard in chloroform, and dilute quantitatively and stepwise with chloroform to obtain a standard solution having a known concentration of about $5\text{ }\mu\text{g}$ per ml. Concomitantly determine the absorbances of both solutions in 1-cm cells at the wavelength of maximum absorbance at about 252 nm, with a suitable spectrophotometer, using chloroform as the blank. Calculate the quantity, in mg, of $\text{C}_{14}\text{H}_{12}\text{O}_3$ in the Trioxsalen taken by the formula $10C(\text{Au}/\text{As})$, in which C is the concentration, in μg per ml, of USP Trioxsalen Reference Standard in the Standard solution, and Au and As are the absorbances of the solution of Trioxsalen and the Standard solution, respectively.

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ERRATA FOR VOLUME 9

CEFAMANDOLE NAFATE

Rafik H. Bishara and Eugene C. Rickard

Page 145, Section 6.3, line 3
Reference (45) should be (44)

Page 145, Section 6.4, line 2
Reference (44) should be (45)

Page 146, Section 6.5.3, bottom line
Reference (45) should be (44)

Page 147, Section 6.6.2, last reference
Reference (44) should be (45)

FLUPHENAZINE DECANOATE

Geoffrey Clarke

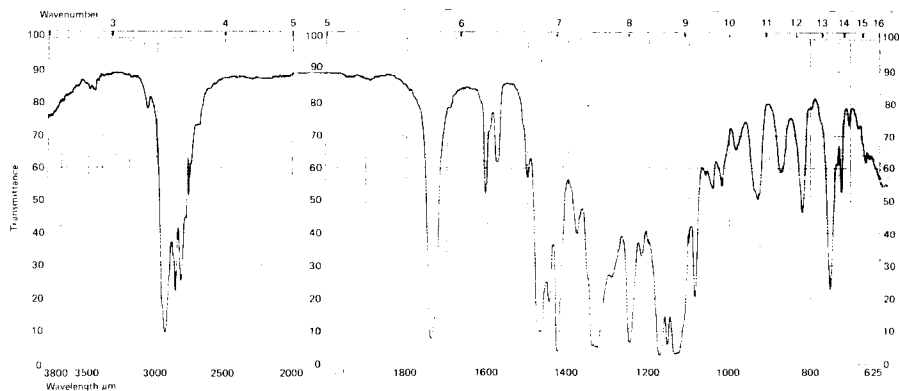


Figure 1. Infrared spectrum of Fluphenazine decanoate as a thin film. Instrument: Unicam SP 1000.

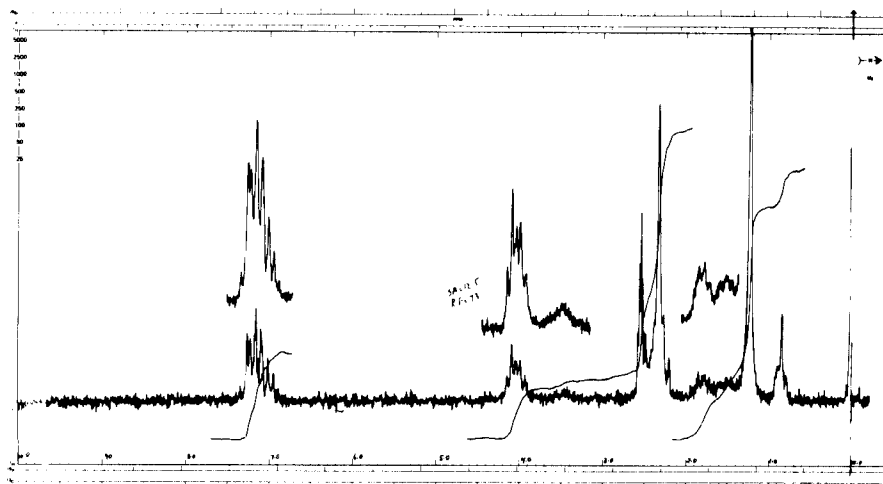


Figure 3. Nuclear magnetic resonance spectrum of Fluphenazine decanoate in DMSO-d₆. Instrument: Thompson Packard.

GENTAMICIN SULFATE

*Bernard E. Rosenkrantz, Joseph R. Greco,
John G. Hoogerheide, and Edwin M. Oden*

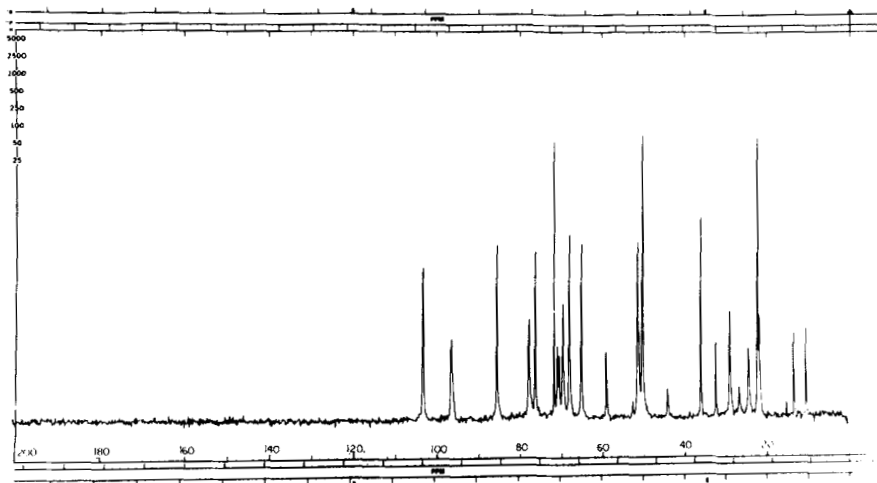


Figure 4. Carbon-13 NMR spectrum of Gentamicin sulfate USP Reference Standard.

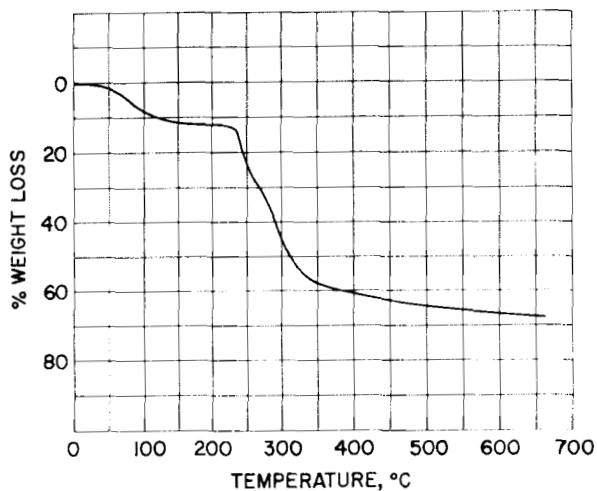


Figure 6. Thermogravimetric analysis curve (TGA) of Gentamicin sulfate USP Reference Standard.

NADOLOL

Lidia Slusarek and Klaus Florey

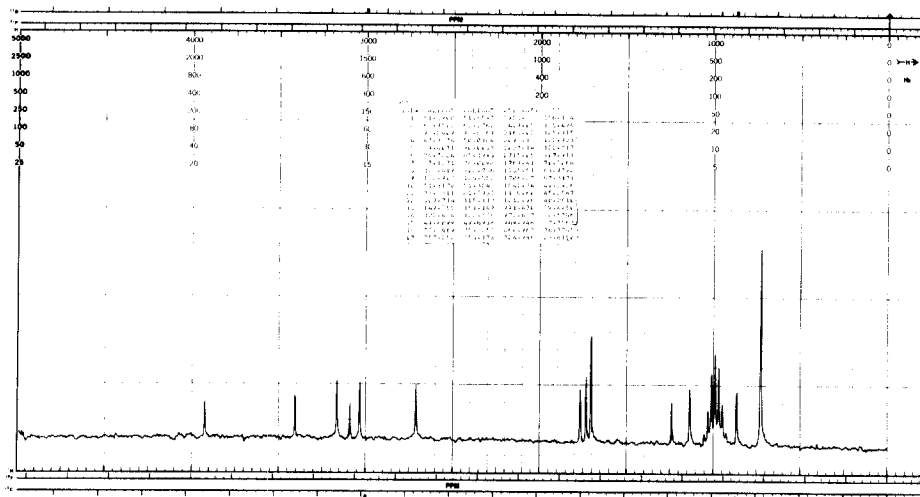


Figure 5. ^{13}C NMR spectrum of Nadolol in $\text{DMSO}-d_6$. Instrument: Varian XL-100-15 operated at 25.2 MHz.

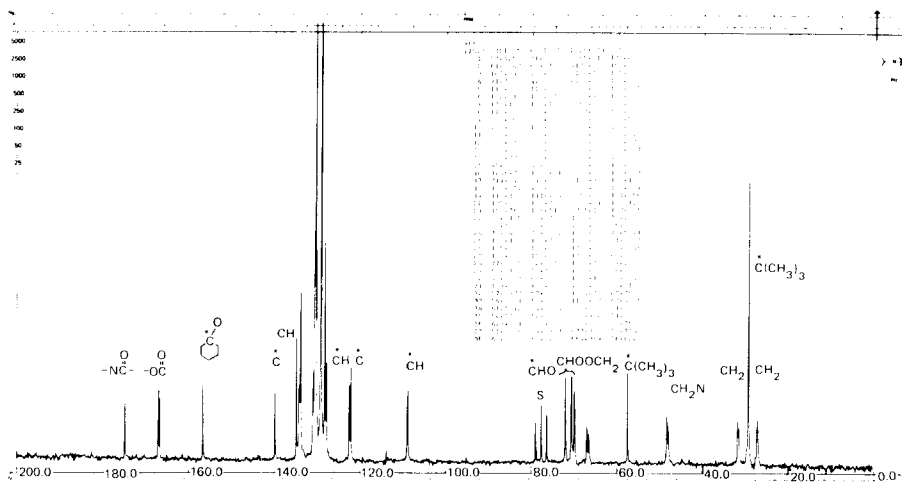


Figure 6. ^{13}C NMR spectrum of Tetrabenzoate derivative of Nadolol in CDCl_3 . Instrument: Varian XL-100-15, operated at 25.2 MHz.

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